ANTIOXIDANT ENZYMES AND RELATED MECHANISMS IN MALIGNANT PLEURAL MESOTHELIOMA

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ABSTRACT

Malignant pleural mesothelioma is a rare but fatal tumor caused mainly by asbestos exposure. There is no standard treatment as mesothelioma is primarily resistant to all treatments including chemotherapy. Asbestos-induced oxidative stress is thought to play an essential role in the pathogenesis of mesothelioma in the process possibly increasing the expression of the major antioxidant defense mechanisms of the cells. Both chemo- and radiotherapy act at least partly by provoking reactive oxygen species (ROS) generation suggesting a role for the intracellular antioxidants in drug resistance. Other mechanisms associated with drug resistance include the plasmamembrane drug transporters, of which several are also redox-regulated.

In the present study, the expression and possible role of the major antioxidant enzymes (AOEs), i.e. manganese superoxide dismutase (MnSOD), catalase, and mechanisms closely related to glutathione (GSH) metabolism were investigated in the biopsies of malignant mesothelioma and/or cell lines in culture. The methods included Northern Blotting, Western Blotting analysis, immunohistochemistry and measurement of specific enzyme activities. Cell damage after oxidant or cytotoxic drug exposures was analyzed by lactate dehydrogenase release, depletion of high-energy nucleotides and microculture tetrazolium dye assay.

MnSOD was highly expressed in mesothelioma tumor biopsies *in vivo* and cell lines *in vitro* compared to non-malignant mesothelial cells. Mesothelioma cell line expressing the highest MnSOD (10 fold compared to non-malignant mesothelial cells) levels also had the highest levels of GSH, glutathione S-transferase (GST) and catalase, and was the most resistant cell line to oxidants and cytotoxic drugs.
In contrast to mesothelioma cells, lung A549 adenocarcinoma cells, that represent an oxidant and drug resistant cell line, contained similar levels of MnSOD as non-malignant mesothelial cells. They, however, also contained higher intracellular GSH levels and catalase than mesothelioma cells, and also had elevated levels of γ-glutamylcysteine synthetase (γGCS), the rate-limiting enzyme in GSH biosynthesis. In contrast to tumor necrosis factor-α (TNFα), cytotoxic drugs failed to induce MnSOD mRNA, protein or activity in A549 cells. Endogenous level of MnSOD or its induction by TNFα did not explain oxidant resistance of these cells. GST could not explain the resistance of adenocarcinoma cells, as the activity of total GST was lower in adenocarcinoma cells than in more sensitive mesothelioma cells.

The role of GSH and catalase was also investigated by treating the mesothelioma cells and A549 adenocarcinoma cells with buthionine sulfoximine (BSO), to block glutathione synthesis, and aminotriazole (ATZ) to inhibit catalase. Both BSO- and ATZ-treatment enhanced H_2O_2 toxicity in three mesothelioma cell lines, while only the depletion of glutathione increased epirubicin toxicity. BSO treatment also significantly potentiated cisplatin-induced cytotoxicity in mesothelioma and adenocarcinoma cells.

Given the obvious importance of GSH in the oxidant and drug resistance of these tumors, altogether 34 mesothelioma tumor biopsies were investigated for both subunits of γGCS. The catalytic, heavy subunit of γGCS was highly expressed in most of the cases, whereas the regulatory, light subunit (γGCSl) expression was weaker. No expression of these proteins could be detected from the non-malignant mesothelium.

The integral membrane drug transporter, P-glycoprotein (P-gp), immunopositivity was found in 61 %, and multidrug resistance proteins 1 and 2 (MRP1 and MRP2) immunopositivity in 58 % and 33 % of 36 mesothelioma biopsies. Normal mesothelium did not express these multidrug resistant proteins. There was no significant association between tumor proliferation, apoptosis or patient survival and expression of the multidrug resistant proteins.

In conclusion, a simultaneous induction of multiple antioxidant enzymes can occur in human mesothelioma cells. In addition to the high MnSOD activity, H_2O_2-scavenging antioxidant mechanisms, γGCS, GST and GSH can partly explain the high oxidant and drug resistance of these cells *in vitro*; the role of catalase during heavy oxidant exposure is possible. MnSOD can be induced by TNFα, but the induction, however, does not provide any protection against repeated oxidant exposures. Many mechanisms contributing to the resistance of mesothelioma remain to be investigated, but γGCS may play important role in the primary drug resistance of this tumor *in vivo* in maintaining the intracellular glutathione-level. The multidrug resistance proteins P-gp, MRP1 and MRP2 are expressed in mesothelioma cells, but are not likely to be responsible for the primary drug resistance of this malignancy.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals.

I. Kahlos, K., Anttila, S., Asikainen, T., **Kinnula, K.**, Raivio, K.O., Mattson, K., Linnainmaa, K., Kinnula, V.L. (1998). Manganese superoxide dismutase in healthy human pleural mesothelium and in malignant pleural mesothelioma. *Am J Respir Cell Mol Biol*, 18, 570-580.

II. **Kinnula, K.**, Linnainmaa, K., Raivio, K.O., Kinnula, V.L. (1998). Endogenous antioxidant enzymes and glutathione-S-transferase in protection of mesothelioma cells against hydrogen peroxide and epirubicin toxicity, *Br J Cancer*, 77, 1097-1102.

III. **Järvinen, K.**, Pietarinen-Runtti, P., Raivio, K.O., Linnainmaa, K., Kinnula, V.L. (2000). Antioxidant defense mechanisms of human mesothelioma and lung adenocarcinoma cells. *Am J Physiol Lung Cell Mol Physiol*, 278(4), L696-702.

IV. **Järvinen, K.**, Soini, Y., Kinnula, V.L. γ-Glutamylcysteine Synthetase in Lung Cancer; Effect on Cell Viability. Driscoll B (ed) *Series in Molecular Medicine: Lung Cancer*. In Press.

V. **Järvinen, K.**, Soini, Y., Kahlos, K., Kinnula, V.L. Overexpression of γ-glutamylcysteine synthetase in human malignant mesothelioma. *Submitted.*

VI. Soini, Y., **Järvinen, K.**, Kaarteenaho-Wiik, R., Kinnula, V. The expression of P-glycoprotein and multidrug resistance proteins 1 and 2 (MRP1 and MRP2) in human malignant mesothelioma. *Annals of Oncology*. In press.

ABBREVIATIONS

ABC	ATP binding cassette superfamily
AOEs	antioxidant enzymes
ATZ	aminotriazole
BSO	buthionine sulfoximine
CAT	catalase
CPM	counts per minute
CuZnSOD	copper-zinc superoxide dismutase
ECSOD	extracellular superoxide dismutase
G6PDH	glucose-6-phosphate dehydrogenase
γGCS	γ-glutamylcysteine synthetase
GSH	reduced glutathione
GPx	glutathione peroxidase
GR	glutathione reductase
GS	glutathione synthase
GSSG	oxidized glutathione
GST	glutathione S-transferase
γGT	γ-glutamyl transpeptidase
HPF	high power field
Kb	kilobase
Kd	kilodalton
LDH	lactate dehydrogenase
LPR	lung resistance protein
MDR	multidrug resistance
MnSOD	manganese superoxide dismutase
MRP	multidrug resistance protein
MT	metallothionein
NSCLC	non small cell lung cancer
NF-κB	nuclear factor -κB
$NO^{\cdot}$	nitric oxide
P-gp	P-glycoprotein
ROS	reactive oxygen species
RNS	reactive nitrogen species
SCLC	small cell lung cancer
SOD	superoxide dismutase
PCR	polymerase chain reaction
SV40	simian virus 40
TGFβ	transforming growth factor-β

TNFα	tumor necrosis factor-α
TRX	thioredoxin
TRXR	thioredoxin reductase
TS	thymidylate synthase
XTT	microculture tetrazolium assay
VEGF	vascular endothelial growth factor

INTRODUCTION

Mesothelioma is a tumor derived from the serosal lining of the pleural, peritoneal or pericardial cavities and is most commonly situated in the pleura. Mesotheliomas are rare tumors, accounting for only about 1% of all cancer deaths in the world (Hammar, 1994). Pleural mesothelioma is in approximately 85-90% of cases an asbestos-initiated lethal malignancy (Mossman et al., 1990). The latency period is about 20 to 40 years. Accordingly, the peak in mesothelioma cases is expected in 2010, although the asbestos usage in most industrialized countries has been abolished from the 1980's. The prognosis of mesothelioma is poor, as it is highly invasive and primarily resistant to all treatments including radiotherapy and cytotoxic drugs. A major factor in the pathogenesis has been considered asbestos-induced oxidative stress, which in turn is known to induce several antioxidant mechanisms in the cells (Janssen et al., 1993). Mesothelioma provides an important model for cancer research of a therapy-resistant malignancy in which antioxidant mechanisms may at least partly explain the resistance.

Intracellular antioxidants offer protection not only against reactive oxygen species (ROS) but may also modulate the response to different chemotherapeutic drugs that are used in cancer treatment. Manganese superoxide dismutase (MnSOD) that scavenges superoxide radicals has a controversial role in cancer biology. It has been suggested to be a cancer suppressor (Oberley & Oberley, 1997) but on the other hand it offers protection against oxidative stress and thereby may confer resistance against oxidant producing drugs. MnSOD is overexpressed in only some malignant tumors, but its importance in drug resistance is unsolved (Cobbs et al., 1996; Janssen et al., 1998; Nishida et al., 1993).

Glutathione has in many studies been linked with drug resistance both for its role as an antioxidant but also for its function in detoxification reactions (Tew, 1994). In most recent studies, attention has been drawn to the enzymes in glutathione biosynthesis and how the cell maintains its glutathione level (Rahman & MacNee, 2000). Studies have also been done with other mechanisms that utilize intracellular glutathione and transport it extracellularly (Borst et al., 2000; Keppler, 1999).

Glutathione S-transferases are a family of detoxification enzymes that are often associated with chemoresistance (O'Brien & Tew, 1996; Tew, 1994). However, the activity of these enzymes is unknown in mesothelioma even though polymorphism of GSTM1 has been linked to the development of this disease (Hirvonen et al., 1996).

Catalase in addition to glutathione takes part in scavenging excess hydrogen peroxide in the cells. Not many studies link it to drug resistance of malignant cells (Sinha & Mimnaugh, 1990), but its role should be clarified in oxidant and drug resistance of mesothelioma.

The classical inducers of multidrug resistance are the drug export pumps in the plasmamembrane that have different substrate specificities. P-glycoprotein has been studied most, but the recently discovered MRP family offers new avenues for investigators in cancer biology (Borst et al., 2000;

Pastan & Gottesman, 1987). The first members in the MRP family, MRP1 and MRP2, are dependent on intracellular glutathione and they transport glutathione-conjugated substrates. In mesothelioma, these mechanisms have not been thoroughly studied before.

This series of studies was designed to systematically investigate the expression of the most important antioxidant pathways and drug transporters in mesothelioma cells *in vitro* and tumor biopsies *in vivo.* Besides investigating the expression of these mechanisms, their role in oxidant and chemotherapeutic drug resistance was assessed *in vitro*. The expression of the AOEs and related proteins was also correlated with tumor growth and patient survival.

REVIEW OF THE LITERATURE

Malignant pleural mesothelioma

History

In 1767 J. Lieutaud recognized two pleural tumors in a series of autopsies, but Wagner was the first to describe the pathology of a primary malignant pleural tumor in 1870 (reviewed by Browne, 1995). The term mesothelioma was first used by Eastwood and Martin in 1921(Boutin et al., 1998). In 1960 Wagner (Wagner et al., 1960) reported 33 cases of diffuse pleural mesothelioma in South Africa, in an area of crocidolite mining. Of these 33 patients, 32 had a history of asbestos exposure, and this connected mesothelioma with asbestos. The first reports of mesothelioma in Finland are from the 1960's (Karjalainen et al., 1997).

Epidemiology

Mesothelioma is a rare disease, but its incidence keeps increasing despite the industrial restriction of asbestos usage from 1980's, as the latency period is approximately 20-40 years. About 70 cases of mesothelioma are diagnosed in Finland every year (Mattson et al., 1999). It has been estimated that the peak of mesothelioma incidence in Finland will be around 2010, with approximately 100 cases per year (Karjalainen et al., 1997). The peak incidence has been already achieved in the U.S, but e.g. in Britain the number of cases per year is climbing and is expected to increase to more than 3000 cases per year (Boutin et al., 1998; BTS, 2001). Mesothelioma is more common among men, only about 10% occur in women. In about 80-90% of the male cases an obvious asbestos exposure is known. In females, it has been suggested that only 23% of mesothelioma cases are asbestos-related (Attanoos & Gibbs, 1997). Sporadic cases among children and infants occur.

Etiology

Asbestos is the single most important causative agent of mesothelioma, and the exposure to asbestos fibers is usually occupational (Craighead & Mossman, 1982). Other lung diseases are caused by asbestos as well, including asbestosis, lung cancer, pleural plaques, pleural fibrosis, pleural effusions and pseudotumors (Mattson, 2000). Factors determining the risk of mesothelioma include the fiber type, time from exposure, fiber dimensions and fiber surface properties (Jaurand et al., 1987; Mossman et al., 1996). There is evidence that persons with a greater intensity and duration of asbestos exposure have a higher risk for mesothelioma which, however, can develop with minimal exposure. Therefore, the causative role of asbestos is difficult to rule out as most adults in the industrialized world have asbestos in their lungs. Fibers greater than 8 μm in diameter are most commonly associated with mesothelioma (Attanoos & Gibbs, 1997).

Asbestos is a commercial term for a variety of naturally occurring hydrated fibrous silicates (Mossman et al., 1996). The material is subdivided into two groups, serpentine fibers and amphiboles. The capacity of different types of asbestos fibers to induce mesothelioma seems to be greatest with amphiboles like amosite (“brown asbestos”) and crocidolite (“blue asbestos”), whereas the serpentine fiber chrysotile (“white asbestos”) is not as tumorigenic (Boutin et al., 1996).

Chrysotile comprises 90% of the asbestos used worldwide. In Finland, however, the main asbestos used has been anthophyllite, which is one of the amphiboles. It is associated with asbestos-induced diseases such as asbestosis and pleural plaques. Mesothelioma cases are rare but some have been reported (Karjalainen et al., 1994). Non-asbestos causes of mesothelioma have not been revealed in epidemiological studies, but theoretically any agent injuring pleura may cause mesothelioma. These include chemical agents, chronic inflammation, viruses and radiation. Smoking does not increase the risk for mesothelioma (Rudd, 1995). Recently a possible connection to Simian Virus 40 (SV40) was suggested (Carbone et al., 1994). In the late 1950’s and the early 1960’s polio vaccines were contaminated with SV40 and millions of people were exposed. In Finland vaccines were not contaminated and none of the mesothelioma patients in Finland had received a contaminated vaccine. SV40 large T-antigen has been detected in a high proportion of mesothelioma tissue specimens. However, other SV40-like DNA sequences were also found in non-malignant pleural diseases. The role of SV40 is unclear, event though in the U.S. and many parts of Europe the consensus seems to link it to mesothelioma.

Genetic susceptibility is associated at least with some detoxification enzyme polymorphisms, including the homozygous deletion of GSTM1 gene or slow acetylation-associated N-acetyl transferase-2 (NAT2) genotype (Hirvonen et al., 1995).

ASBESTOS

Serpentine

CHRYSOTILE
$Mg_6Si_4O_{10}(OH)_8$

Amphiboles

CROCIDOLITE
$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$

AMOSITE
$(Fe,Mg)_7Si_8O_{22}(OH)_2$

TREMOLITE
$Ca_2Mg_5Si_8O_{22}(OH)_2$

ANTHOPHYLLITE
$(Mg,Fe)_7Si_8O_{22}(OH)_2$

ACTINOLITE
$Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_2$

Figure 1. Classification of asbestos fibers (modified from Mossman et al., 1996)

Pathogenesis and pathology

The inhaled asbestos fibers must be transported to the pleural cavity to reach the target cells (Browne 1995). Parietal pleura is often more extensively involved, but usually it is difficult to determine if mesotheliomas begin in the visceral or parietal pleura (Attanoos & Gibbs, 1997). When inhaled into the respiratory bronchioles and alveoli, chrysotile fibers are usually fragmented by organic acids and cleared by macrophages. Amphiboles are not as easily decomposed and may remain unchanged for years/decades. The asbestos fibers are transported to the pleural cavity via the lymphatic pathway or by penetrating to the visceral pleura. Amphibole fibers concentrate on certain areas of the parietal pleura, called black spots, that are openings of lymph vessels, and at these spots the pleura is exposed for years to the effects of asbestos fibers and toxic reactive oxygen species (ROS) (Boutin et al., 1996). Free radicals and other toxic oxygen metabolites are considered important in the pathogenesis of mesothelioma (Mossman et al., 1989). Fibers themselves have redox properties as they contain ferrous iron which catalyses the reaction forming ROS (Kamp et al., 1992). ROS are also formed indirectly when phagocytic cells meet the fibers; macrophages and neutrophils are known to liberate ROS after asbestos exposure (Klockars & Savolainen, 1992; Hedenborg & Klockars, 1987; Nyberg & Klockars, 1990). These active oxygen intermediates can participate in the oncogenic process by many different mechanisms. Genotoxicity, lipid peroxidation, and oncogene modulation are all possible effects of ROS. The long latency period suggests cumulative genetic, cytotoxic and proliferative events (Janssen et al., 1993).

Pleural mesothelioma is divided histologically into three classes (Travis et al., 1999). The epithelial subtype comprises about 54% of all mesothelioma cases. Epithelial mesotheliomas may be predominantly composed of acinar structures, and differential diagnosis from adenocarcinoma is often demanding. Other variants of epithelial mesothelioma also exist. Sarcomatotic mesotheliomas, that histologically resemble fibrosarcomas, represent approximately 20% of the cases, and the rest of the cases fall into biphasic mesotheliomas, representing about 25% of the cases (Hammar, 1994).

Clinical features and diagnosis

The average age of a patient at the time of diagnosis is approximately 60 years, and there is a strong male predominance (Mossman & Gee, 1989). The first symptoms include chest pain, dyspnea, weakness and cough. Usually the diagnosis is delayed due to the non-specificity of the symptoms. Thoracic radiograph initially shows pleural effusion in 92% of cases, usually on one side. Only in 7 % a multinodular pleural tumor without fluid is seen (Boutin et al., 1998). In early cases of mesothelioma, nodules or plaques of varying size can be detected in the parietal pleura. Serosal thickening and consequent effusion is often marked. The majority of cases are unicavitary. Mesothelioma seldom sends metastasis, but it is highly invasive, e.g. to the pericardium.

One of the first diagnostic procedures is cytology of pleural fluid that gives positive results in approximately 30% of cases. Another method used for a diagnostic workup is the computed tomography (CT) scan. The diagnosis is established by biopsy via thoracoscopy in most of the cases. Examination of biopsy of parietal and visceral pleura is the most reliable method for diagnosis (BTS, 2001).

Histological diagnosis is, however, difficult because of structural variability between different tumors and even within the same tumor, the main problem being differential diagnosis from metastatic adenocarcinoma of the lung. Other differential diagnostic difficulties arise from bening mesothelial hyperplasia and sarcomas in cases of sarcomatoid mesothelioma. In addition to the typical histopathology, panel of immunohistochemical stains will often suggest the right diagnosis. Many antigens stain positively in adenocarcinoma but remain negative in mesothelioma. The markers used in the diagnostic procedure include the carcinoembryonic antigen (CEA), glycoprotein markers Leu-M1, Ber-EP4 and B72.3, and others like epithelial marker antigen (EMA) and human milk fat globulin-2 (HMFG-2). In epitheloid tumors, diastase resistant neutral mucin is positive in approximately 70% of adenocarcinomas, but usually negative in epithelial mesothelioma. In case of sarcomatoid mesothelioma cytokeratins like CK 5/6 and AE1/AE3 are used, as they are generally positive in sarcomas and negative in sarcomatoid mesothelioma. Calretinin, that reveals the mesothelial origin, is usually positive in mesothelioma and negative in sarcoma and its specificity is over 90% (King & Hasleton, 2001). In differentiating between reactive and neoplastic mesothelium attention should be focused on the degree of cellular atypia and the presence of collagen necrosis that are highly suggestive of malignancy.

Table 1. Immunohistochemistry of pleural lesions (modified from Travis et al., 1999).
LMW, low molecular weight; HMW, high molecular weight; HMGF, human milk fat globulin; CEA, carcinoembryonic antigen; EMA, epithelial membrane antigen; Vim, vimentin.
-, negative; +/- occasionally positive; -/+, usually negative; +, usually positive.

Diagnostic problem	Keratins LMW/HMW	CEA	B72.3	Leu-M1	BER-EP4	EMA HMFG-2	Vim
Mesothelial hyperplasia vs.	+	-	-	-	-	-/+	-/+
Epithelial mesothelioma vs.	+	-/+	-/+	-/+	-/+	+	+
Metastatic carcinoma (adeno ca)	+	+	+	+	+	+	-/+
Fibrous pleuritis vs.	+	-	-	-	-	-/+	+
Sarcomatoid mesothelioma vs.	+	-	-	-	-	-/+	+
Sarcoma (primary or metastatic)	-/+	-	-	-	-	-	+

Treatment and prognosis

Treatment of malignant mesothelioma remains disappointing, and there is no standard treatment (BTS, 2001). As in other malignant tumors, surgery, radiation therapy, chemotherapy, supportive therapy or a combination of different modalities are used. No treatment has so far been shown to offer better survival than supportive therapy alone. Median survival time from diagnosis is less than one year, 5-year survival is less than 5 %. Some factors, however, indicate a more favorable prognosis, including epithelial subtype, age < 65 years, good clinical condition with no weight loss, and absence of visceral pleura involvement (Hammar, 1994).

Surgery alone does not improve survival but may be beneficial for palliation. Four different surgical methods are in use: extrapleural pneumonectomy, pleurectomy/decortication, limited pleurectomy and thoracoscopy with talc pleurodesis. Extrapleural pneumonectomy is often used in the combination with radiotherapy.

Radiotherapy is also used for palliation, especially in cases with pain. Sometimes the disease may regress, but significant improvement in survival has not been achieved. Radiotherapy is usually given in combination with either surgery or chemotherapy, so the individual effects of the treatment modalities are difficult to document.

Many different chemotherapeutic agents have been tested either as single-agent treatment or in combination therapy. In the best clinical series, objective responses are seen after single-agent therapy in about 20-30% of patients, but no significant effect on the overall survival (BTS, 2001). The best results in single-agent treatment have been achieved using anthracyclins, with doxorubicin giving up to a 40% response rate and high-dose cisplatin a response rate of up to 33%. Rather promising results have been achieved also with carboplatin, epirubicin, ifosfamide and mitomycin. High-dose methotrexate treatment resulted in a response rate of 37% in a study of 63 patients (Boutin et al., 1998). Combinations of cisplatin, doxorubicin or an alkylating agent like ifosfamide have been studied, usually two or three drugs are combined. No clear advantage over single-agent therapy has been observed. Combination therapy with cytokines, like interferon-α, has been disappointing despite the promising results in *in vitro* studies (Boutin et al., 1998). The resistance mechanisms of mesothelioma tumors have been studied only in few publications and therefore remain largely unknown. Some of these studies will be discussed later.

Lung cancer

Given the difficulties between the differential diagnosis of mesothelioma and lung cancer, this study has included experiments also on the biopsies and cell lines of lung cancer, mainly lung adenocarcinoma.

The incidence of lung cancer is increasing due to the habit of tobacco smoking in the world. Over 3 million lung cancer deaths have been estimated worldwide in the year 2000. In Finland 2 075 new lung cancer patients were diagnosed in 1994, after five years only 10% are still alive (Mattson, 2000). Lung cancer is the second most common cancer among men in Finland. The incidence among women is climbing and at present lung cancer is the second most common cause of cancer deaths among women.

Tobacco is the most important etiological agent of all four subtypes of lung cancer responsible for approximately 90% of all cases. Other known exogenous risk factors for lung cancer include asbestos, ionizing radiation, and other environmental carcinogens e.g. polycyclic aromatic hydrocarbons, nitrosamines and aromatic amines. The endogenous, host related factors, include immunological factors and genetic predisposition, mainly differences in carcinogen metabolism, DNA repair and altered proto-oncogene and/or tumor suppressor gene expression (Vainio & Husgafvel-Pursiainen, 1996).

Lung cancer is divided into two major classes mainly for treatment purposes: small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). Virtually all cases arise from the epithelial tissue and are bronchogenic carcinoma subtypes. SCLC (30% of all lung cancers) proliferates fast, often sends metastases and is primarily sensitive to anti-cancer drugs. Therefore, the initial treatment is chemotherapy. However, resistance to treatment develops rapidly and many different resistance mechanisms have been speculated. P-glycoprotein cannot solely explain the clinical drug resistance (Lai et al., 1989) and other possible drug resistance mechanisms include multidrug resistance proteins (Wright et al., 1998) and decreased expression of topoisomerase II (Giaccone, 1994).

NSCLC comprises three histologically different carcinomas: adenocarcinoma (30-35%), squamous cell carcinoma (30-35%), and large cell anaplastic carcinoma (5%) (Mattson, 2000). The treatment of NSCLC is primarily surgery. Combination chemotherapies are widely used for the treatment of

NSCLC since only 10-20% of the cases can be operated. In contrast to SCLC, NSCLC is primarily resistant to single chemotherapeutic agents. In adenocarcinoma, glutathione-related mechanisms have been suggested as potential resistance inducers along with other classical resistance mechanisms (Giaccone et al., 1996; Oguri et al., 1998a; Oguri et al., 1998b; Scagliotti et al., 1999; Sugawara et al., 1995; Zhang et al., 1998).

Reactive oxygen and nitrogen species

A free radical is defined as a chemical species that has a single unpaired electron in the outer orbital (Fridovich, 1978). In this state the radical is extremely reactive and unstable. The most important radicals are the superoxide radical ($O_2^{-\cdot}$), the hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$). Reactive oxygen species (ROS) include free radicals and other oxygen-related reactive compounds, such as hydrogen peroxide (H_2O_2) (Halliwell, 1991). ROS are generated in normal aerobic metabolism in mitochondria, which are the main site of production of radicals. In the cytosol and plasma membrane, ROS are formed by NADPH oxidase, cytochrome P450 oxidase and xanthine oxidase (see Figure 3). Transitional metals, such as iron and copper, are potential promoters of free radical damage, as they can convert superoxide, which in normal conditions is poorly reactive, into a rapidly reactive and highly toxic hydroxyl radical by Fenton chemistry (Halliwell & Gutteridge, 1985). In Haber-Weiss reaction, hydroxyl radical is generated from $O_2^{-\cdot}$ and H_2O_2. $NO^{\cdot}$ has many useful physiological functions, but in excess amounts is a toxic free radical as well. Many exogenous agents, such as hyperoxia, radiation, asbestos fibers and ozone induce free radical formation in the cell. Asbestos fibers cause oxidant production directly and indirectly, one of the ways being catalysis by the ferrous ion, as asbestos fibers have a high iron content. Inflammatory cells, such as neutrophils and alveolar macrophages, also produce large amounts of ROS when activated, especially when the phagocytosis is incomplete (Kamp et al., 1992). $NO^{\cdot}$ production is also activated via the induction of inducible nitric oxide synthase by TNFα and other cytokines released from the inflammatory cells. Reactive nitrogen species that are formed in reactions of $NO^{\cdot}$ and oxygen/superoxide mediate the harmful effects of $NO^{\cdot}$ (Ohshima & Bartsch, 1994).

The pathological effects of ROS are wide-ranging; these toxic products can cause injury practically to all cellular components. Lipid peroxidation of membranes, non-peroxidative mitochondrial damage, lesions in DNA, and cross-linking of proteins are the most relevant reactions of ROS leading to cell injury. ROS are thought to be especially important in lung tissue that is exposed to much higher concentrations of oxygen than most other tissues, but also to cigarette smoke and environmental pollutants. In addition to the toxic effects, ROS are important in non-toxic cellular reactions, including signal transduction (Thannickal & Fanburg, 2000).

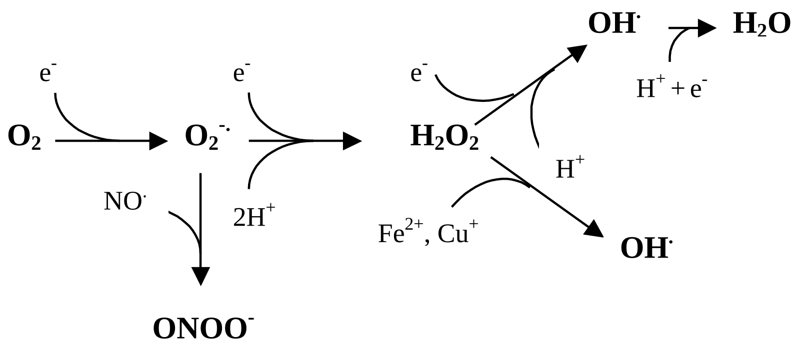

Figure 2. The pathway of oxygen reduction and peroxynitrite formation

Antioxidants

To protect themselves from the harmful effects of oxidants, cells have several antioxidant enzymes and other antioxidant mechanisms. The latter include glutathione (GSH) and numerous GSH-dependent enzymes, metal binding proteins, and vitamins. The three main types of antioxidant enzymes are the superoxide dismutases (SODs), catalase (CAT) and peroxidases, of which glutathione peroxidases (GPx) are thought to be the most important (Halliwell and Gutteridge 1989). The SODs dismutate the superoxide radical into H_2O_2. GPx and CAT reduce H_2O_2 into water and oxygen. Glutathione redox cycle provides the cell with reduced glutathione (GSH) to act as cosubstrate for the peroxidases but to also participate in detoxification reactions and react non-enzymatically with $OH^{\cdot}$ and peroxynitrite. Other enzymes involved in glutathione metabolism are glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione S-transferases and the enzymes participating in GSH-synthesis: γ-glutamylcysteine synthetase (γGCS) and glutathione synthase (GS). Metal-binding proteins ferritin, ceruloplasmin, transferrin, haptoglobin and albumin contribute to the antioxidant system by inactivating catalytic metals. The most important antioxidant vitamins include α-tocopherol, ascorbate, B-carotene and flavonoids, but they will not be discussed in this review.

Other enzymes with antioxidant capacity include cysteine-containing proteins such as the families of thioredoxin, glutaredoxin and peroxiredoxin. These may play a role in the resistance of cells against oxidants but also against free radical generating drugs (Holmgren, 2000; Powis et al., 2000; Rhee et al., 1999).

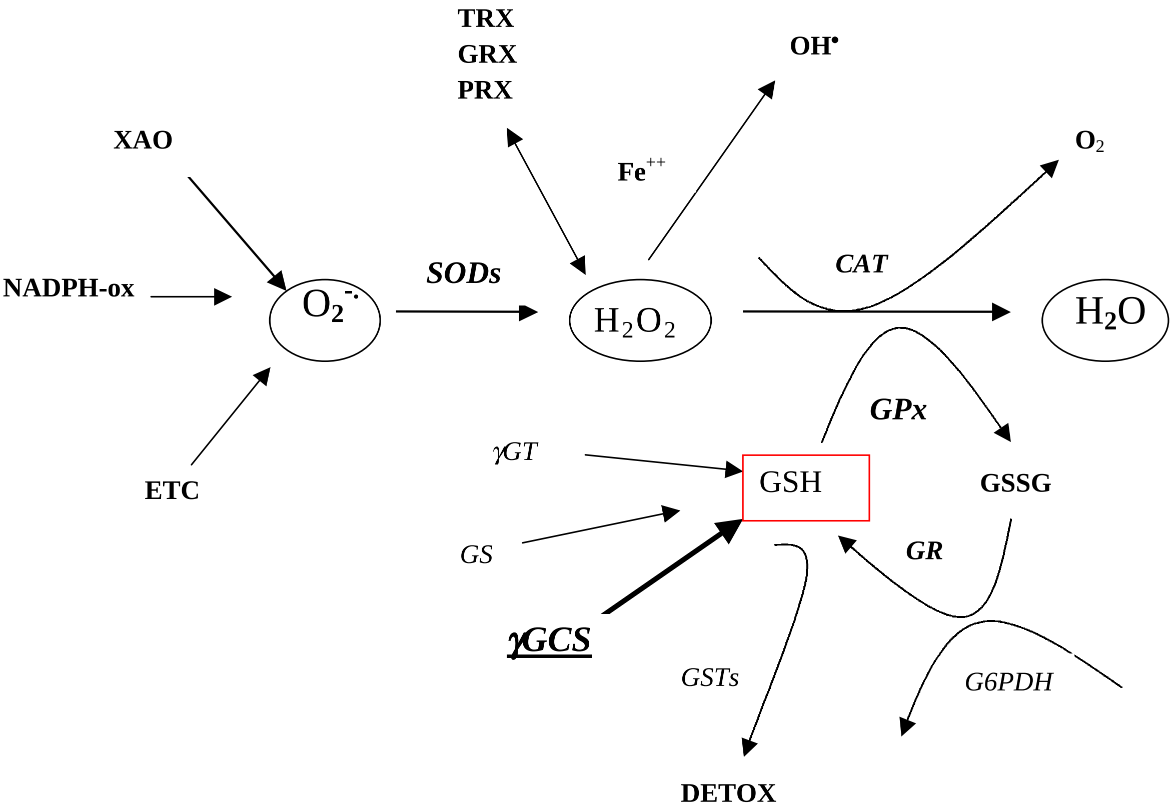

Figure 3. The central intracellular antioxidant mechanisms in mammalian cells. XAO = xanthine oxidase, NADPH-ox = NADPH-oxidase, ETC= mitochondrial electron transport chain, TRX = thioredoxin, GRX = glutaredoxin, PRX = peroxiredoxin, SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, GR = glutathione reductase, GSH = glutathione, GSSG = oxidized glutathione, GST = glutathione S-transferase, γGCS = γ-glutamylcysteine synthetase, GS = glutathione synthase, γGT = γ-glutamyl traspeptidase, G6PDH = glucose- 6-phosphate dehydrogenase

Superoxide dismutases

Two main forms of SOD exist intracellularly: a copper-zinc containing superoxide dismutase (CuZnSOD) and a manganese-containing superoxide dismutase (MnSOD) (Fridovich, 1995). CuZnSOD is found in the cytoplasm, and MnSOD in the mitochondria. Extracellular SOD (ECSOD) is located in the extracellular matrix.

MnSOD (also known as SOD2) is a homotetramer with a molecular weight of 88 000 and is located in the mitochondrial matrix close to the electron transport chain, where ROS are produced in normal cellular metabolism (Fridovich, 1998). The gene is located in the long arm of chromosome 6 and is transcribed as two distinct mRNAs of 1 kb and 4 kb (Church et al., 1992). MnSOD is synthesized in the cytoplasm as a precursor molecule containing a leader signal, which is removed during the transport of the molecule to the mitochondria (Wan et al., 1994; Weisiger & Fridovich, 1973). Two polymorphic variants of MnSOD have been described, one leading to altered mitochondrial targeting of the enzyme and the other possibly to changed MnSOD *in vitro* activity (Ho & Crapo, 1988; Rosenblum et al., 1996). The importance of MnSOD for normal physiology has been proven with knockout mice lacking the MnSOD gene, who died within 10-20 days of neurological manifestations and cardiotoxicity (Li et al., 1995). Heterozygous mice with half of the MnSOD activity have increased age-related mitochondrial oxidative damage (Williams et al., 1998). Approximately 15% of total intracellular SOD activity is due to MnSOD.

In eukaryotic cells, the MnSOD gene regulation is complex. The MnSOD promoter contains binding sites for several transcription factors such as AP1, AP2, SP1 and NF-κB. It has been hypothesized that the oxidative state of the cell is essential in regulating MnSOD expression. MnSOD is induced by the cytokine tumor necrosis factor α (TNFα) (Wong & Goeddel, 1988). TNFα binds to its plasma membrane receptor, which initiates a series of events including intracellular ROS production, activation of NF-κB and induction of the MnSOD gene. The TNFα induction of MnSOD is blocked by the antioxidant N-acetyl cysteine (NAC). Other factors that induce MnSOD are hyperoxia, irradiation, oxidized LDL, interleukin-1, interferon-γ, lipopolysaccarides, H_2O_2 and asbestos fibers (Crapo & Tierney, 1974; Harris et al., 1991; Oberley et al., 1987; Visner et al., 1990; Warner et al., 1996; Wong & Goeddel, 1988). In some studies the MnSOD gene induction is associated with resistance to hyperoxia, which would indicate that oxidant stress induces the enzyme to protect from subsequent oxidant injury (Liochev & Fridovich, 1997; Tsan et al., 1990; Wispe et al., 1992). However, in contrast to many *in vivo* hyperoxic models, MnSOD is not directly upregulated by high oxygen tension in human bronchial epithelial cells *in vitro* (Pietarinen-Runtti et al., 1998). In human lung MnSOD is found in type II pneumocytes, bronchial epithelial cells and alveolar macrophages (Coursin et al., 1996; Kinnula et al., 1994; Lakari et al., 1998). High levels of MnSOD are also found from the heart, brain, liver and kidneys (Beyer et al., 1991).

In human malignancies, the role of MnSOD is controversial. In carcinogenesis, the antioxidant – oxidant imbalance is considered significant. A polymorphism of the MnSOD gene resulting in alteration in the transport of MnSOD into the mitochondria due to conformational change in the protein is a risk factor at least for the development of breast and lung cancers (Wang et al., 2001); Ambrosone et al., 1999). Most studies have shown that MnSOD activity is low in cancer cells, and it has been proposed to be a cancer suppressor gene (Oberley & Oberley, 1997). Transfection studies, in which only the MnSOD gene has been introduced, have shown decreased level of malignancy and transformation of the malignant phenotype to the direction of a non-malignant one. However, interpretation of this study is problematic as transfection creates imbalanced conditions in the cell. On the other hand, at least gliomas, thyroid carcinomas, esophageal carcinomas and colon carcinomas appear to contain high MnSOD levels when compared to the non-malignant tissues. In a study of five samples of lung tumors the activity of total SOD was somewhat lower than in normal lung tissue (Jaruga et al., 1994). In mesothelioma, MnSOD has not been previously

studied before our group reported elevated activity of MnSOD in mesothelioma cell lines (Kinnula et al., 1996).
It has been reported that MnSOD is not inducible in cancer cells as it is in non-malignant cells (Wong & Goeddel, 1988) but also this issue is controversial. At least human lung adenocarcinoma cells show MnSOD induction by TNFα (Warner et al., 1996). Human A549 lung cells also represent a malignant cell type but appear to show MnSOD induction (Das & White, 1997).

CuZnSOD (SOD1) is a homodimer with a molecular weight of 32000 and is localized mainly in the cytosol, but it is also found in the nucleus and peroxisomes (Crapo et al., 1992; Fridovich, 1998). The gene is located in chomosome 21, the gene is transcribed as two mRNAs, 0.9 and 0.7 kb, respectively, the latter being the predominant form (Sherman et al., 1984). In contrast to MnSOD, CuZnSOD-deficient animals and cells are viable but they are sensitive to oxygen toxicity (Huang et al., 1999). Mutation of this gene is associated with familial amyotrophic lateral sclerosis (Rosen et al., 1993).
The regulation of CuZnSOD also differs from MnSOD, e.g. its level is constitutive in several animal studies (Clerch & Massaro, 1993) and human lung (Lakari et al., 1998), neither is it induced by hyperoxia (Pietarinen-Runtti et al., 1998; Visner et al., 1990), TNFα or interleukin-6 (reviewed by Kinnula et al., 1995). In healthy human lung, CuZnSOD is found from the bronchial epithelium (Coursin et al., 1996; Lakari et al., 1998). High levels are also found from the liver, erythrocytes, brain and neurons. In a recent study CuZnSOD gene was found to be upregulated in a mesothelioma cell line compared to a non-malignant mesothelial cell line, when assessed in microarray containing over 6900 genes (Rihn et al., 2000). Otherwise its expression and role in human tumors remain unclear.

ECSOD is a copper and zinc containing homotetrameric glycoprotein. It is located in the extracellular matrix in all human tissues and its gene is located in chromosome 4 (Fridovich, 1998; Hendrickson et al., 1990). ECSOD is induced by cytokines like TNFα (Stralin & Marklund, 2000), direct oxidant stress does not affect ECSOD like it does MnSOD. In healthy lung, ECSOD is concentrated in pulmonary vessels and airways, and also found from systemic arteries (Oury et al., 1994). Of the pulmonary cell types, it is found from bronchial epithelium, alveolar macrophages and endothelial cells (Oury et al., 1994). Its role and regulation in cancer are unknown.

Glutathione

Glutathione (L-γ-glutamyl- L- cysteinylglycine, GSH) is the predominant intracellular low molecular weight thiol in all mammalian cells (Meister, 1983), usually present in the millimolar range; the intracellular level being approximately 1-8 mM and the extracellular level typically 5-50 μM (Griffith, 1999). About 99% of the intracellular glutathione is in the reduced form. Approximately 85% of the intracellular glutathione is in the cytosol, about 15% in the mitochondria and a small percentage in the endoplasmic reticulum. The mitochondrial GSH pool is maintained by the activity of a mitochondrial transporter that translocates cytosolic GSH into mitochondria (Rahman & MacNee, 2000).
GSH is a central protective antioxidant against free radicals and other oxidants, but it has also an essential role in detoxification reactions. Other cellular events where glutathione is considered

valuable are modulation of redox-regulated signal transduction, regulation of cell proliferation, remodeling of extracellular matrix, apoptosis, mitochondrial respiration and a reservoir of cysteine (Rahman & MacNee, 2000). Numerous studies show that resistant human cancer cell lines contain high glutathione levels *in vitro* and that oxidant induced toxicity can be enhanced by buthionine sulfoximine (BSO) that causes glutathione depletion by inhibiting its synthesis (Tew, 1994; Zhang et al., 1998; Griffith, 1999; O'Brien & Tew, 1996; Sinha & Mimnaugh, 1990). The role of glutathione in oxidant and drug resistance has not been previously investigated in mesothelioma.

Enzymes in the glutathione redox cycle: Glutathione peroxidase (GPx) and glutathione reductase (GR)

GPx is one of the major enzyme families in removing hydrogen peroxide generated by, e.g., superoxide dismutases. It catalyzes the reaction where GSH is oxidized into GSSG and H_2O_2 converted into water and oxygen. Four distinct selenoproteins are included in the family of glutathione peroxidases, the classical form being the cytosolic GPx, which is also found in the mitochondria and extracellular matrix. The other three are the gastrointestinal form of GPx (Chu et al., 1993), a non-selenium dependent GPx (Shichi & Demar, 1990) and phospholipid hydroperoxide GPx (Schuckelt et al., 1991).

The cytosolic GPx, a tetrameric selenoprotein, has a molecular weight of 85 000. The gene is located in chromosome 3 (Moscow et al., 1994). Recently a polymorphism was found that associated to lung cancer (Ratnasinghe et al., 2000). In normal physiological conditions with low or moderate production of H_2O_2, GPx has been considered a more important scavenger than catalase, because its Michaelis-Menten constant (Km value) for H_2O_2 is lower than that of catalase. Selenium is needed in the synthesis of GPx and at least the extracellular GPx is induced by hyperoxia and oxidants (Avissar et al., 1989; Erzurum et al., 1993).

GPx is ubiquitously expressed in erythrocytes, kidney and liver. The expression of GPx in malignant tumors is somewhat variable. GPx activity has been suggested to be elevated in adenocarcinoma of the lung (Carmichael et al., 1988; Di Ilio et al., 1987) whereas it was decreased in other lung cancer subtype biopsies when assessed by immunohistochemistry (Coursin et al., 1996; Jaruga et al., 1994). Elevated GPx activity has been linked with chemoresistance of anti-cancer drugs, such as adriamycin, that kills cells by releasing free radicals (reviewed by Sinha & Mimnaugh, 1990).

Glutathione reductase (GR) converts GSSG back to GSH at the expense of NADPH forming a redox cycle (see Figure 4). Two isoenzymes of GR, one cytosolic and one mitochondrial, are encoded by a single gene located in chromosome 8. It has been postulated that the glutathione conjugates formed in xenobiotic detoxification can inhibit GR thereby accumulating GSSG altering the redox capacity of the cell. The expression of GR in human lung and tumors is unclear.

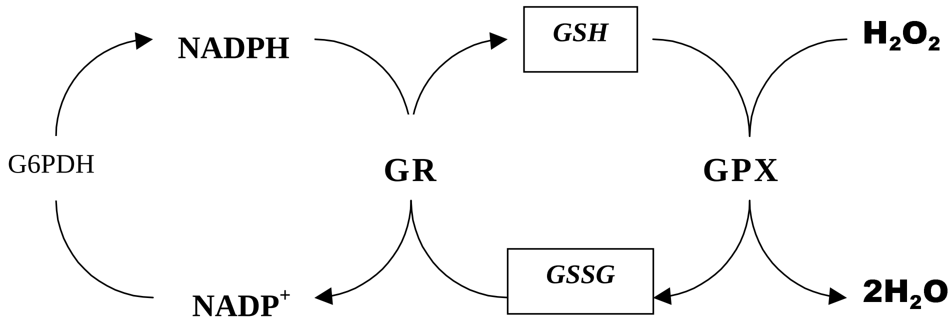

Figure 4. The glutathione redox cycle. G6PDH = glucose-6-phosphate dehydrogenase, GR = glutathione reductase, GSSG = oxidized glutathione, GPx = glutathione peroxidase and GSH = glutathione.

Enzymes in glutathione biosynthesis

γ-glutamylcysteine synthetase (γGCS) is the rate-limiting enzyme in GSH biosynthesis (Richman & Meister, 1975). The synthesis requires another ATP-dependent enzyme, glutathione synthase and the amino acids glutamic acid, cysteine and glycine. In general, the activity of γGCS defines the rate of glutathione synthesis and γGCS is feedback-inhibited by the product, GSH. Cysteine is the rate-limiting substrate. Levels of GSH and cysteine are the two factors that regulate the synthesis of glutathione under physiological conditions. The importance of glutathione synthesis was proven in a recent study which showed that homozygous knockout mouse lacking the γGCS heavy subunit gene dies before birth (Dalton et al., 2000).

γGCS is a cytosolic heterodimer consisting of a heavy subunit (γGCSh, MW~ 73 000) and a light subunit (γGCSl, MW ~30 000) (Seelig et al., 1984). γGCSh gene is located in chromosome 6 (6p12) and γGCSl gene in chromosome 1 (1p21) and two mRNA transcripts are consistently seen for both subunits (Gipp et al., 1995). γGCSh is the catalytically active subunit; it also binds the feedback inhibitor GSH. It has been suggested that γGCSh alone comprises about half of the enzyme activity when compared with the holoenzyme (Mulcahy et al., 1995). Some studies, however, have concluded that it has no catalytic activity without the light subunit (Lu et al., 1999). γGCSl serves an important regulatory role and reduces the inhibitory effect of GSH. It has been suggested that during GSH depletion, in oxidizing conditions, the enzyme undergoes conformational changes between subunits that allows an increase in the enzyme activity. In normal physiological conditions when abundant amounts of GSH are present, both subunits are needed for the enzyme activity (Huang et al., 1993).

γGCS is induced by several agents, including oxidants e.g. H_2O_2 and menadione, cytokines e.g. TNFα, heavy metals e.g. cadmium and iron, and some chemotherapeutic agents e.g. cisplatin (Lu, 1999). At transcriptional level γGCS subunits are regulated by a number of regulatory signals, including ARE, TRE, AP1 and NF-κB. γGCS activity is also regulated at the post-transcriptional and translational level, and phosphorylation/dephosphorylation may control its activity. Possible inhibitors include glucocorticoids, insulin, prostaglandin E (Rahman & MacNee, 2000), and TGF-β (Arsalane et al., 1997). Exposure to sublethal doses of oxidants may initiate an adaptive antioxidant response, where the intracellular GSH is first depleted leading to oxidant stress and consequent γGCS upregulation. The role of γ-glutamyl transpeptidase (γGT) in regulating γGCS activity indirectly by cleaving extracellular GSH has also been suggested (Hanigan, 1998). The expression of γGCS mRNA varies between different tissues. In healthy human lung, γGCS mRNA has been detected from bronchial epithelial cells (Rahman et al., 1999).

There are no previous studies on the expression or distribution of γGCS in malignant tumors. It has been suggested that as chromosome 1 (loss of 1p21-22) is often deleted in malignant mesothelioma, this would predispose an individual to the development of the tumor (Rozet et al., 1998). Elevated levels of γGCS have been detected in many drug-resistant malignant cell lines. Chemoresistance may be associated with accumulation of GSH, which functions as an antioxidant but is also used in detoxification reactions. Glutathione has also been shown to inhibit apoptosis by changing the redox state of the cell (Manna et al., 1998). Apoptosis resistance in turn has been considered important in the drug resistance of malignant cells.

Glutathione synthase (GS) is a cytosolic homodimer that catalyses the reaction of L-γ-glutamyl-L-cysteine and glycine that forms GSH. GS is composed of two apparently identical subunits (each MV~52 000) and the gene is located in chromosome 20 (Webb et al., 1995). Two forms of glutathione synthetase deficiency have been described. One form is mild, causing hemolytic anemia, but the other more severe form causes 5-oxo-prolinuria with secondary neurological involvement (Webb et al., 1995). The regulation of GS is poorly known.

In glutathione biosynthesis, the availability of cysteine is crucial. Cysteine is transported into the cell by a sodium-dependent A system and cystine, an oxidized form of cysteine, by an inducible transporter Xc^- (Bannai, 1984). Cystine is then reduced to cysteine that can be used in GSH biosynthesis. The transport of cystine is induced by oxidants, such as hyperoxia and H_2O_2, contributing to increased GSH levels during oxidative stress (Deneke & Fanburg, 1989). There are no studies on the expression of GS or cysteine transporters in malignant tumors.

γ-glutamyl transpeptidase (γGT) acts as a salvage enzyme in GSH synthesis. The molecular weight is 50 kD for the heavy and 25 kD for the light subunit (Arai et al., 1995). The gene is located in chromosome 22 (Figlewicz et al., 1993). γGT is located on the plasmamembrane, where it cleaves the γ-glutamyl bond in extracellular γ-glutamyl cysteinyl-glycine (Hanigan & Ricketts, 1993). The amino acids are returned into the cell and reused for GSH synthesis. γGT is induced by menadione and t-butyl hydroquinone (Liu et al., 1998), suggesting its role in protecting cells during oxidative stress. In addition to other luminal surfaces of the body, lung epithelium contains high levels of this enzyme (Ingbar et al., 1995). There is one study showing that mesothelioma biopsies are negative for this enzyme when assessed by immunohistochemistry (Hanigan et al., 1999). In the same study, strong immunoreactivity is detected from renal cell carcinoma, adenocarcinoma of the prostate and papillary carcinoma of the thyroidea (Hanigan et al., 1999).

Glutathione S-transferases (GSTs)

GSTs are a superfamily of detoxifying enzymes that have broad substrate specifities (Hayes & Pulford, 1995). Five families of cytosolic GSTs have been identified in human, of which four have been thoroughly characterized: Alpha (α), Mu (μ), Pi (π) and Theta (τ). The genes for GST-μ class are all located in chromosome 1, whereas GST-π gene is in chromosome 11. A polymorphism of GSTM1 (μ-class) resulting in dysfunction of the enzyme has proven to be a risk factor for malignant diseases, including mesothelioma (Hirvonen et al., 1996). The GSTs conjugate GSH with compounds containing an electrophilic center and thereby provide critical protection against xenobiotics and products of oxidative stress. Since the GSH-conjugate is transported out of the cell, intracellular GSH is consumed irreversibly in the conjugation and thus maintenance of intracellular GSH levels is essential for the optimal function of GSTs. Many GST enzymes possess GPx activity as well. Many of the substrates of GSTs also induce the expression of the GST genes, suggesting an adaptive response to chemical stress. Carcinogens and alkylating agents may induce GST-π (Zhang et al., 1998).

The GST-π family is the predominant GST in human solid tumors and has even been used as a marker in lung, colon, bladder and other human cancers (Zhang et al., 1998). GST activity is often associated with anticancer drug resistance, as the drugs are converted to a less toxic form by the conjugation. Based on one study 77% of mesothelioma cell lines expressed GSTπ in immunohistochemistry (Dejmek et al., 1998).

Catalase

Catalase (CAT) is a tetrameric hemoprotein that catalyses the reaction of decomposition of H_2O_2 into water and oxygen. It has a molecular weight of 240 000. It is mainly localized in the peroxisomes (Davies et al., 1979) but is also found in the cytoplasm and mitochondria in minor amounts. The gene is localized in chromosome 11. Patients suffering from acatalasemia have a mutation of the CAT gene but are clinically healthy. Catalase has a higher K_m than GPx, which suggests a major role for CAT at higher levels of H_2O_2 but a minor role at physiological levels of H_2O_2 (Halliwell & Gutteridge). Catalase is not abundantly present in the mitochondria, where the physiological oxidative stress is at its highest. It has been shown to be induced by high oxygen tension in alveolar epithelial cells (Freeman et al., 1986). In other studies, however, no induction could be detected in lung epithelial cell after oxidant or cytokine exposures (Erzurum et al., 1993; Pietarinen-Runtti et al., 1998).

There are no systematic studies on catalase in malignant tumors. Some studies have suggested variable catalase expression in lung, breast and colon cancers (Cable et al., 1992; Coursin et al., 1996; el Bouhtoury et al., 1992). One recent study showed that catalase is highly expressed in mesothelioma (Kahlos et al., 2001b). No major role has been suggested to catalase in drug resistance (Sinha & Mimnaugh, 1990).

Other proteins with antioxidant capacity

Glutaredoxin and peroxiredoxins are cysteine-containing H_2O_2-scavenging proteins, that have been recently described (Holmgren, 2000; Rhee et al., 1999), but no investigations of these proteins have been conducted in human lung tumors. Thioredoxin is composed of two closely related cysteine-containing proteins, thioredoxin (TRX) and thioredoxin reductase (TRXR). This group of proteins enhances cell proliferation and increases resistance to apoptosis in several *in vitro* and *in vivo* experimental models (Powis et al., 2000). There are two recent studies showing overexpression of TRX and TRXR in lung tumors and mesothelioma (Kahlos et al., 2001a; Soini et al., 2001a). However, the expression of these proteins did not correlate with survival in either tumor.

Heme oxygenase (heat shock protein 32) has also been shown to have antioxidative properties. There are no systematic studies on this enzyme in malignant tumors. Based on unpublished studies from the Department of Internal Medicine, University of Oulu, it is not overexpressed in mesothelioma.

Metallothioneins (MT) have been proposed as possible inactivators of metal-containing chemotherapeutic agents intracellularly. MT's contain a high level of cysteine and they have the ability to bind heavy metal ions. MT content has been found to correlate with the resistance of SCLC cell lines to cisplatin (Kasahara et al., 1991). MT is expressed in approximately half of mesothelioma tumor biopsies, but does not correlate with patient survival (Isik et al., 2001).

ATP-dependent multidrug transporters

Drug efflux glycoproteins are the most studied mechanisms in primary/acquired cytotoxic drug resistance (Bellamy & Dalton, 1994; Kartner et al., 1983; Ling, 1992). These pumps are ATP-dependent, located in the plasmamembrane, and offer resistance to a wide variety of drugs by decreasing their net cellular accumulation. Two separate families are known, namely P-glycoproteins encoded by the MDR1 gene and the MRP-family of proteins.

P-glycoprotein

P-glycoprotein is a 170-kDa mammalian ATPase and it belongs to a large superfamily of integral membrane transport proteins, called the ATP Binding Cassette (ABC) superfamily (Doige et al., 1993; Higgins, 1992). It is encoded by the MDR1 gene located in chromosome 7, and two classes (classes I and III) of P-gp exist in humans (Childs & Ling, 1994). P-gp is normally expressed in detectable quantities at least in colon, adrenal cortex, kidney and liver. The blood-brain barrier has a very high expression level of P-gp, which is necessary to restrict the entrance of various drug molecules into the central nervous system.

P-gp is the most studied multidrug resistance mechanism in human cancer. Increased rate of drug efflux and in some cases also decreased rate of drug influx result in decreased intracellular drug accumulation. It has been suggested that in about 50% of human cancers the MDR1 gene is expressed at levels that are thought to be significant (Goldstein, 1995). P-gp is known to transport agents such as anthracyclins, vinca-alkaloids, taxol and epipodophylotoxins (Volm, 1998). The MDR1 gene has been shown to be regulated by heat shock, arsenite and cadmium as well as other cytotoxic compounds (Chaudhary & Roninson, 1993; Chin et al., 1990). Different mechanisms of how the pump actually works have been suggested. P-gp has been mostly studied in hematological malignancies because of easy availability of tissue. The general conclusion is that at least in acute myelogenous leukemia, P-gp probably has a role in the development of chemoresistance (Campos et al., 1992).

The significance of P-gp in solid tumors is less clear. In kidney, liver and colon carcinomas the expression of P-gp has been associated with shorter survival (Duensing & Slate, 1994; Sinicrope et al., 1992; Soini et al., 1996; Verrelle et al., 1991). In adenocarcinoma of the breast the prognostic value of P-gp screening remains unclear as controversial results have been obtained (Huang et al., 1998; Trock et al., 1997; Verrelle et al., 1991). The role of P-gp in other solid tumors, as in urologic tumors, may be significant (van Brussel & Mickisch, 1998). However, it has been suggested that it may not play role in non-small cell lung carcinoma, as only very low or undetectable levels of the MDR1 gene product are expressed in these tumors (Lai et al., 1989). In mesothelioma, one immunohistochemical study showed that majority of the cases expressed P-gp (Ramael et al., 1992). However, its role in mesothelioma remained unclear as in a study of five doxorubicin resistant mesothelioma cell lines expression of MDR1 gene could not be detected. (Ogretmen et al., 1998).

MRP family

In addition to P-gp, another family of transporter proteins, multidrug resistance proteins, has been characterized (Cole et al., 1992). While only two genes encode the P-gps, many more genes seem to be related to the MRP family. Currently the family has eight members (Bera et al., 2001; Borst et al., 2000).

MRP1 (MW 190 000) was the first member of the family. It shares a 15 % amino acid identity with the P-gp and its gene is located in chromosome 16 (Cole et al., 1992). Physiologically, MRP1 transports leukotriene 4 (Borst et al., 2000). MRP1 knockout mice are viable, but their response to an inflammatory stimulus is impaired (Wijnholds et al., 1997).

Preferred substrates for MRP1 are organic anions and drugs conjugated to glutathione, glucuronate or sulfate. MRP1 has proven to be the previously characterized glutathione S-conjugate pump. It has been stated that MRP-mediated export of conjugates represents an indispensable terminal step in detoxification, and the co-ordinate overexpression with the detoxification enzyme GST leads to high level resistance to cytotoxic drugs (Keppler, 1999) (see Figure 4). MRP1 transports vinca alkaloids and anthracyclins conjugated to glutathione, and therefore depletion of intracellular glutathione results in reversal of the drug resistance (Borst et al., 2000). In many cell lines a simultaneous increase in the expression of MRP1 and γGCS is often detected when exposed to pro-oxidants such as menadione and heavy metals like cadmium and arsenite (Ishikawa et al., 1996; Kuo et al., 1998; Kuo et al., 1996). MRP1 has also been linked to the oxidative state of the cell, as oxidative stress enhances the expression of MRP1 in cultured cells (Yamane et al., 1998). Cisplatin resistance has not been seen in MRP1 overexpressing cells.

MRP1 is found ubiquitously in the human body; in non-malignant lung tissue MRP1 has been detected in bronchial epithelium and hyperplastic pneumocyte II cells; (Wright et al., 1998) (Thomas et al., 1994).

Most histological subtypes of NSCLC, but not SCLC, have detectable levels of MRP, when assessed with immunohistochemistry; (Nooter et al., 1998; Sugawara et al., 1995; Wright et al., 1998). In cancers such as breast cancer (Nooter et al., 1997), gastric cancer (Endo et al., 1996), retinoblastoma (Chan et al., 1997), NSCLC (Young et al., 2001) and neuroblastoma (Norris et al., 1996) MRP1 expression has been associated with drug resistance or poor patient outcome. In five mesothelioma cell lines, MRP1 was found to be overexpressed when compared to non-malignant mesothelial cells (Ogretmen et al., 1998). MRP1 tissue expression has not been previously studied in mesothelioma.

MRP2 was previously known as the canalicular multispecific organic anion transporter that is in normal physiological conditions found in the canalicular membrane of hepatocytes (Borst et al., 2000; Paulusma et al., 1996). It has a 45 % amino acid identity with MRP1 and the gene is located in chromosome 10 (Borst et al., 2000). Patients with Dubin-Johnson syndrome have inactivating mutations in their MRP2 gene and are deficient in their bilirubin–glucuronide secretion (Paulusma et al., 1996). In addition to liver, MRP2 is found from kidney and gastrointestines.
MRP2 in known to handle a similar range of GSH conjugates as MRP1. The expression of MRP2 in tumor tissues is at present unclear. MRP2 expression in lung cancer cells has not been thoroughly studied; in colorectal cancer the mRNA levels of MRP2 correlated with resistance to cisplatin (Hinoshita et al., 2000). MRP2 is also expressed in gastric tumor cell lines (Narasaki et al., 1997). MRP2 mRNA seems to be present in cultured cell lines as well as samples of patient tissues with no difference between NSCLC and SCLC (Young et al., 1999). In transfected cells, overexpression of MRP2 results in resistance to methotrexate, cisplatin, etoposide, doxorubicin, epirubicin and mitoxantrone (Borst et al., 2000; Cui et al., 1999; Konig et al., 1999).

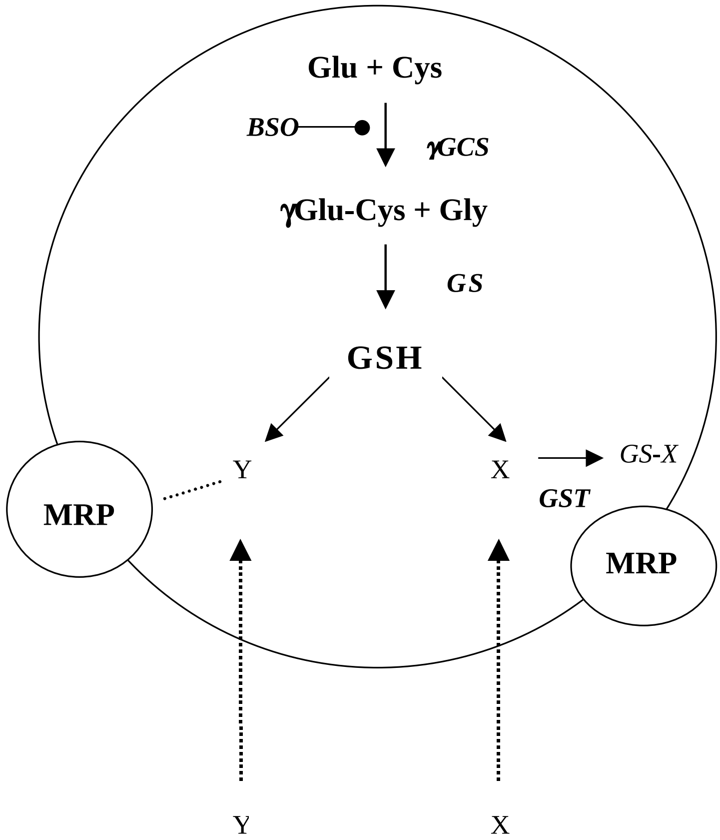

Figure 5. Model showing the relationship between MRP and glutathione. Some drugs (X) conjugated to glutathione by GSTs are then transported by MRP. Others (Y) are cotransported with glutathione. In both cases, transport is glutathione dependent and can be blocked with BSO. BSO = buthionine sulfoximine, γGCS = γ-glutamylcysteine synthetase, GS = glutathione synthase, GSH = glutathione, GST = glutathione S-transferase, GS-X = glutathione conjugate, MRP = multidrug resistance protein

Other mechanisms of cytotoxic drug resistance

Resistance to chemotherapy represents a major source of failure in cancer treatment. Resistance can be roughly divided into two classes: primary (intrinsic) resistance, as in mesothelioma and NSCLC, and acquired resistance that develops rapidly during treatment, as in SCLC. The mechanisms behind these two classes are, however, overlapping. The resistance mechanisms naturally vary between drugs that have different mechanisms of action. Most of the information on drug resistance has been obtained from anthracyclins and alkylating agents. Anthracyclins are redox-active antibiotic chemotherapeutics and form quinone-hydroquinone structures, when activated. The most commonly used anthracyclins are epirubicin, daunorubicin and adriamycin. Menadione and mitomycin C are also quinones that are used in anticancer treatments and form ROS. The use of anthracyclins is often limited in clinical practice because of difficult side effect including cardiotoxicity. Alkylating agents include cisplatin, carboplatin and ifosfamide. These agents bind to DNA interfering with proliferation of the cell. Cisplatin forms intrastand cross-links in DNA, especially in-between neighboring guanines. Cisplatin causes renal failure as a side effect due to necrosis of tubular cells and is highly emetic.

The most studied drug-resistance mechanism is, as already mentioned, P-gp. However, other mechanisms besides P-gp, MRPs and glutathione conjugation may be involved. Recently, one mechanism of interest has been resistance to apoptosis, and the expression of the bcl-2 group of proteins such as bcl-2, bcl-XL, mcl-1 and bax are widely investigated. The expression and significance of various bcl-2 group proteins are, however, unclear in many cancers, but it is known that bcl-2 is associated with decreased and bax with increased apoptosis. Mesothelioma has low or undetectable bcl-2 expression and weak to moderate expression of bcl-X, mcl-1 and bax. However, the expression levels do not appear to correlate with apoptosis of the tumor or prognosis of this disease (Soini et al., 1999). Another oncogene, p53, is associated with enhanced apoptosis and widely investigated in malignant tumors (Thompson, 1995). The expression of p53 does not correlate with survival of mesothelioma patients, either (Esposito et al., 1997).

Chemotherapy has been generally agreed to be most successful in rapidly proliferating tumors, and therefore many cell cycle proteins have been thought to be involved in drug resistance (Drewinko et al., 1981). Topoisomerase II is a ubiquitous nuclear enzyme that is important in DNA functioning, replication, recombination and transcription. Decreased activity confers “atypical” resistance to e.g. anthracyclins and epipodophyllotoxins in human leukemic cells (Beck et al., 1993). A recent study with five mesothelioma cell lines suggested that mutations in topoisomerase IIα can be associated with resistance of mesothelioma to etoposide (McLaren et al., 2001). Lung resistance protein (LPR), localized in the perinuclear area, is one of four vault proteins which form barrel-like structures and its overexpression has been associated with decreased drug response at least in acute myeloid leukemia and ovarian carcinoma (Scheffer et al., 1995). In mesothelioma its expression has not been studied.

Thymidylate synthase (TS) plays a central role in DNA biosynthesis and is the target of many chemotherapeutic drugs. TS expression has been connected to doxorubicin resistance in lung cancer (Volm et al., 1979) but also to resistance to cisplatin (Xu et al., 1991). No studies have been conducted on TS expression in mesothelioma. Many solid tumors contain areas of hypoxic cells that are more resistant to drugs than the cells in normoxia (Teicher, 1994). This can partly be explained by poor vascularisation and hence decreased influx of anti-cancer agents into the hypoxic area. However, it has been found that tumors with poor vascularisation can express at least GSTπ, TS, MT and P-gp (Koomagi et al., 1995). At present the most rapidly growing area of cancer research is undoubtedly angiogenesis of solid tumors (Saaristo et al., 2000). For instance, a recent study on mesothelioma reported the expression of several angiogenic cytokines, such as vascular endothelial growth factor (VEGF), transforming growth factor (TGFβ) and fibroblast growth factor (FGP), in this disease. These cytokines may contribute to the high invasiveness but not to the primary drug resistance of mesothelioma (Kumar-Singh et al., 1999).

AIMS OF THE STUDY

Asbestos fibers provoke oxidative stress, which is thought to play an essential part in the pathogenesis of mesothelioma. Mesothelioma is primarily resistant to chemo- and radiotherapy, both of which act at least partly by provoking ROS generation in the cells. It can be hypothesized that in mesothelioma antioxidant mechanisms may play an important role in the pathogenesis and drug resistance and in part explain the poor prognosis. These mechanisms have not been previously studied in this disease, neither have the potential mechanisms of primary drug resistance, such as the drug export pumps, been explored in mesothelioma. Comparison with lung adenocarcinoma gives further information on the specificity of these mechanisms in malignant disease.

The specific aims were:

1. To study the expression and localization of MnSOD in mesothelioma tissue biopsies compared to non-malignant mesothelium *in vivo* and to assess the expression of this enzyme in mesothelioma cell lines *in vitro*.

2. To investigate *in vitro* the inducibility and role of MnSOD in mesothelioma cells in comparison with adenocarcinoma cells with low MnSOD activity.

3. To investigate the activities of catalase and glutathione-associated pathways in mesothelioma cells and to evaluate their importance in oxidant and drug resistance.

4. To study γGCS expression in lung and mesothelioma tumor biopsies and cell lines, and to assess its significance in primary drug resistance of mesothelioma.

5. To investigate the expression of the multidrug resistance proteins, P-gp, MRP1 and MRP2, in mesothelioma tumor biopsies and to evaluate their role in tumor growth and aggressiveness.

Table 2. Characteristics of the mesothelioma patients

Patient	AGE/SEX	Diagnosis	Survival (months)	Asbestos exposure	Smoking history
1	42/F	Sarcomatoid	3	nk	-
2	56/M	Epithelial	9	+	+
3	57/F	Biphasic	21	nk	-
4	58/M	Sarcomatoid	nk	nk	nk
5	63/M	Epithelial	1	nk	+
6	74/M	Epithelial	124	+	-
7	66/M	Epithelial	0	+	-
8	70/F	Epithelial	2	-	+
9	70/M	Biphasic	14	-	-
10	50/M	Epithelial	7	+	+
11	68/M	Biphasic	2	nk	
12	63/M	Epithelial	9	+	+
13	52/M	Sarcomatoid	2	nk	+
14	33/F	Sarcomatoid	96	nk	-
15	71/M	Sarcomatoid	4	+	+
16	73/M	Epithelial	24	nk	+
17	67/M	Epithelial	8	-	+
18	59/M	Epithelial	8	+	+
19	54/M	Epithelial	1	+	+
20	67/M	Sarcomatoid	4	-	-
21	78/M	Epithelial	21	+	+
22	57/M	Biphasic	21	+	+
23	57/M	Epithelial	3	nk	nk
24	73/M	Sarcomatoid	0	nk	+
25	78/M	Epithelial	1	+	+
26	79/M	Epithelial	1	+	-
27	63/M	Epithelial	5	+	+
28	66/M	Epithelial	6	+	+
29	72/M	Epithelial	34	+	+
30	73/F	Epithelial	40	+	nk
31	68/M	Sarcomatoid	5	-	-
32	69/M	Epithelial	2	-	+
33	69/M	Epithelial	7	+	+
34	77/M	Epithelial	11	+	-
35	52/F	Epithelial	13	nk	nk
36	67/F	Epithelial	*	-	+
37	62/M	Epithelial	13	+	+
38	43/M	Epithelial	7	+	-
39	72/M	Epithelial	20	+	+
40	44/M	Biphasic	5	+	+
41	52/M	Biphasic	14	+	+
42	46/M	Biphasic	13	+	+
43	54/M	Biphasic	6	+	-

nk= not known, *= still alive

MATERIALS

Mesothelioma patients

Altogether 43 biopsies of mesotheliomas were included, 7 biopsies were obtained from the Finnish Institute of Occupational Health (I) and 36 from the Department of Pathology, Oulu University Hospital (V, VI). The clinical data on the mesothelioma patients were obtained from the patient records and are presented in Table 2. The survival time was defined as time from diagnosis until death.

Tissue specimens

Healthy visceral pleural tissue and mesothelioma specimens were obtained from surgical biopsy, lobectomy or pulmonectomy samples. The samples of healthy visceral pleura, verified by light microscopic examination, were obtained from individuals who underwent surgery for reasons other than malignant mesothelioma or other pleural disease. Histopathologic diagnosis of malignant mesothelioma was confirmed, when necessary, in the Mesothelioma Panel of Finland and the tumors were classified into epithelial, sarcomatoid or biphasic according to the criteria from WHO (Travis et al, 1999).

Cell lines

Mesothelioma cell lines

Seven mesothelioma cell lines (M10K, M14K, M24K, M25K, M28K, M33K and M38K) were originally established from the tumor tissue of untreated mesothelioma patients, with the exception of M10K which was established from a mesothelioma metastasis (Pelin-Enlund et al., 1990). This particular patient had received both radio- and chemotherapy prior to surgery. With the exception of M25K, all the patients were known to have been exposed to asbestos. The data on these patients are presented in Table 3. The tumor biopsy was processed not more than two hours after surgery. The specimen was freshly minced with a scalpel and cultured as described by Pelin-Enlund et al (Pelin-Enlund et al., 1990). The continuous cell lines were characterized cytogenetically and the tumorigenity of some cell lines was tested on experimental mice. In order to confirm the karyotype of the cell lines after culturing up to passage 53, the cells were re-characterized to reveal that no major changes had occurred, but the cell lines had remained cytogenetically relatively stable.

Table 3. Characteristics of the tumors from which the mesothelioma cell lines originated. Based on Pelin et al, 1994
[1] per gram dry weight
[2] duration of experiment 52-71 weeks, wit M28K 31 weeks.

Cell line	Histological subtype	Fiber content in lung[1]	Number of chromosomes	Tumor incidence in nude mice[2]
M10K	biphasic	13.0×10^6	38-46	2/5
M14K	epithelial	26.0×10^6	41-45	2/5
M24K	biphasic	11.0×10^6	no data	no data
M25K	biphasic	no data	no data	no data
M28K	epithelial	150×10^6	no data	0/7
M33K	biphasic	4.0×10^6	no data	2/5
M38K	biphasic	6.2×10^6	no data	no data

Non-malignant transformed mesothelial cell line – MeT5A

MeT5A is a non-malignant pleural mesothelial cell line from American Type Culture Collection (Rockville, MD, USA). The cell line was originally established by transfecting mesothelial cells with a plasmid-containing Simian Virus (SV40) DNA. MeT5A cells therefore express the SV40 large T antigen, but are non-tumorigenic with typical mesothelial morphology (Ke et al., 1989).

Adenocarcinoma cell line - A549

A549 cells, which are human lung adenocarcinoma cells, were obtained from American Type Culture Collection (Rockville, MD, USA). The cell line was initially established from human alveolar cell carcinoma, and since it has many pneumocyte II characters (Lieber et al., 1976), it has been used both as a model for adenocarcinoma (Das & White, 1997) and type II pneumocyte (Rahman et al., 1999).

Cell culture conditions

The mesothelioma cells and Met5A cells were cultured in monolayer in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.03% L-glutamine at 37 C° in 5% CO_2 atmosphere. A549 cells were grown in monolayer in F12 nutrient mixture supplemented with 15% FCS.

Antibodies

The antibodies that were used in immunohistochemistry and Western blots are listed in Table 4.

Table 4. The primary and secondary antibodies used in immunohistochemistry (IH) and Western blot (WB). Abbreviations: MnSOD = manganese superoxide dismutase, γGCSh = the heavy subunit of γ-glutamylcysteine synthetase, γGCSl = the light subunit of γ-glutamylcysteine synthetase, P-gp = P-glycoprotein, MRP = multidrug resistance protein, GST-π = glutathione S-transferase-π

Antibody	Source	Concentration	Secondary antibody
MnSOD	Polyclonal, rabbit anti-human (Dr.J.D.Crapo)	1:1000 IH 1:10 000 WB	Swine anti-rabbit 1:2000 Swine anti-rabbit 1:2000
γGCSh	Polyclonal, rabbit anti-human (Dr. T.Kavanagh)	1:1000 IH 1:40 000 WB	Anti-rabbit (Zymed Kit) Donkey anti-rabbit1:50 000
γGCSl	Polyclonal, rabbit anti-human (Dr. T.Kavanagh)	1:1000 IH 1:20 000 WB	Anti-rabbit (Zymed Kit) Donkey anti-rabbit1:50 000
P-gp	Monoclonal, mouse anti-human (JSB1, Alexis Biochemicals)	1:20 IH	Anti-mouse (Zymed Kit)
MRP1	Monoclonal, mouse anti-human (MRPm6, Alexis Biochemicals)	1:50 IH	Anti-mouse (Zymed Kit)
MRP2	Monoclonal, mouse anti-human (Alexis Biocemicals)	1:50 IH	Anti-mouse (Zymed Kit)
Ki-67	Monoclonal, mouse anti-human (Zymed)	1:50 IH	Sheep anti-mouse 1:300
Caspase 3	Polyclonal, rabbit anti-human	1:2000 WB	Sheep anti-mouse
GST-π	Polyclonal rabbit anti-human (BioPrep)	1:5000 WB	Donkey anti-rabbit 1:30 000

METHODS

Pretreatments, oxidant and cytotoxic drug exposures

Buthionine sulfoximine (BSO) is a widely used inhibitor of γGCS for the purpose of depleting intracellular glutathione (Buckley et al., 1991). The final concentration of BSO varied between 0.2 mM (MeT5A, M14K) and 1mM (M38K). These concentrations were tested in preliminary studies in which cell survival and intracellular GSH concentrations were analyzed. The pretreatment time was overnight (16-18h).

Aminotriazole (ATZ) inactivates cellular catalase (Margoliash et al, 1960) Cells were pretreated with 20 (II) or 30 (III) mM ATZ for 60 min. These concentrations were selected on the basis of previous studies (Kinnula et al., 1991) and also in the preliminary experiments of this study (II).

TNFα is a cytotoxic cytokine released from inflammatory cells. It causes NF-kB activation and ROS generation in cells and its effects are numerous (reviewed by Vanden Berghe et al., 2000). The concentration of TNFα was 10 ng/ml and the exposure time 24-48 h.

Menadione is a quinone that creates continuous oxidant stress by forming a redox cycle intracellularly (Frei et al., 1986). The continuous generation of oxidants is considered to provide a better model of physiological/pathological conditions in the cell than a bolus of oxidants. H_2O_2, on the other hand, causes a relatively short oxidant stress and is quickly decomposed intracellularly. The concentrations used were 10-100 μM for menadione (4-48h) (I, II, III) or 0.01-1 mM for H_2O_2 (1-48h) (II, III).

The cytotoxic drugs used in these experiments (I, II, III, IV, V) were epirubicin, cisplatin, vindesin and methotrexate. Epirubicin, an anthracyclin, is activated to free radical state and, in addition, it interacts with molecular oxygen to generate ROS and thus causes oxidant-mediated DNA damage (Sinha & Mimnaugh, 1990). Epirubicin was chosen since it is widely used in the treatment of mesothelioma. The concentrations varied between 0.005 μg/ml- 0.5 μg/ml (0.01-1 μM). Cisplatin is an alkylating agent and has a platinum complex in the *cis*-position. Glutathione-related mechanisms have been linked to resistance against cisplatin (Zhang et al., 1998). In addition to other pulmonary malignancies, cisplatin is also used in the treatment of mesothelioma. Cisplatin concentrations varied between 0.02-2 μM in (III), 20-250 μM (IV) and 10-100 μM (V). Methotrexate is a dihydrofolic acid reductase inhibitor but has been shown to alter also γGCS and GPx activity (Kussmann et al., 1993). The concentration varied between 0.01-1 μM (III). Vindesin interferes with microtubule formation in the mitotic spindle, and mainly P-gp has been suggested to explain resistance against it. The concentration varied between 0.007-1 μM. All exposure times were 48h.

Northern Blot analysis

The cells were scraped into 4 M guanine thiocyanate buffer and the samples were immediately frozen at –70° C. Total RNA was isolated with the acid phenol-chloroform method of

Chomczynski and Sacchi (Chomczynski & Sacchi, 1987). Denaturated RNA samples (10-15 μg) were electrophoresed on 1% agarose gel with 0.36 M formaldehyde. The RNA was transferred onto Hybond-N nylon filters after ethium bromide staining and UV examination to check the loading homogeneity, and cross-linked to the filters by UV illumination. The filters were prehybridised at 58.5° C for more than one hour in a buffer containing 50% deionized formamide, 5 x saline-sodium citrate (SSC), 50 mM sodium phosphate at pH 6.5, 5 x Denhardt's reagent, and 100 μg/ml herring sperm DNA.

The cDNA probes for MnSOD, CuZnSOD, catalase and glutathione peroxidase were kindly provided by Dr. Y.-S. Ho from Wayne State University, USA. Filters were hybridized with ^{32}P-labeled cRNA probes representing human MnSOD (Ho & Crapo, 1988), human CuZnSOD (Halliwell & Gutteridge, 1985), human catalase (Quan et al., 1986) or rat GPx (Ho et al., 1988), each cloned into the pSP65 vector. The probes were purified by NucTrap columns (Stratagene) and added to the prehybridization solution at $2x10^6$ cpm/ml. Hybridization was carried out overnight at 58.5 °C with shaking. The filters were washed thoroughly and autoradiography was performed at –80 °C using Kodak BioMax MR film (Eastman Kodak Co, Rochester, NY, USA). After autoradiography, the filters were hybridized with a β-actin control probe transcribed from pTRI-β-actin plasmid (Ambion, Austin, TX, USA). The enzyme mRNA expressions were quantified relative to actin expression by an X-rite 331 Transmission densitometer.

Western blot analysis

The cells were detached with trypsin, centrifuged, and washed with phosphate buffered saline (PBS), then mixed with the electrophoresis sample buffer and boiled for 5 min at 95 C°. Cell protein was measured by using the Bio-Rad method, and 50 μg (I), 30 μg (II) and 70 μg (V) of cell protein were applied per lane in a 12% sodium dodecyl sulphate-polyacrylamide gel. The gel was electrophoresed for 1.5 h (90 V) at room temperature and the protein was transferred (60 min, 100V) onto Hybond ECL nitro cellulose membranes (Amersham, Buckinghamshire, UK) in a Mini-PROTEAN II Cell (Bio-Rad, Hercules, CA). The blotted membrane was incubated with rabbit antibodies to MnSOD (dilution 1:10 000), GST-π (dilution 1:5000), γGCSh and γGCSl (dilution 1:40 000, 1:20 000) or to caspase 3 (dilution 1:2000), followed by a secondary antibody conjugated to horseradish peroxidase (Amersham) (dilution 1:30 000 (I, II); 1:50 000(V, IV)). MnSOD, GST-π, γGCS and caspase 3 protein were detected by an enhanced chemiluminescense system (ECL, Amersham), and the luminol excitation was imaged on X-ray film (Biomax MR, X-Omat, USA).

β-actin expression was analyzed in MnSOD, GST-π and γGCS Western blots in order to confirm loading homogeneity. The membranes were first stripped of antibodies and reprobed using a monoclonal anti-actin antibody and secondary antibody that was conjugated to horseradish peroxidase. β-actin was detected and imaged as described above.

Enzyme activities

The activities of the antioxidant enzymes were evaluated in MeT5A, M14K, M38K and A549 cells. For the assays the cells were detached with trypsin, pelleted and frozen immediately at –70 °C until analysis. The cells were resuspended in PBS and treated with 1% Triton X-100 to disrupt the cellular organelles.

MnSOD

Total superoxide dismutase activity was assessed with some modifications using the method of McCord and Fridovitch (McCord & Fridovich, 1969). The activity of the enzyme was measured following the decrease in the rate of reduction, in other words the absorbancy change rate at 550 nm, of 12.8 μM cytochrome c in the presence of 0.5 mM xanthine and xanthine oxidase. MnSOD activity was distinguished from CuZnSOD activity by its resistance to 1 mM potassium cyanide. The results are expressed as units per mg of total cellular protein.

Glutathione reductase and glutathione peroxidase

Glutathione peroxidase activity was assessed by measuring NADPH oxidation in the presence of t-butylhydroperoxide, glutathione reductase and glutathione according to Beutler (Beutler et al., 1975). Glutathione reductase activity was determined by measuring the oxidation of NADPH in the presence of oxidized glutathione GSSG (Beutler et al., 1975). Enzyme activities are expressed as units per mg of total cellular protein.

GST

Glutathione-S-transferase was measured spectrophotometrically using 1mM 1-chloro-2,4-dinitrobenzene and 1mM glutathione (Habig & Jakoby, 1981). Enzyme activity is expressed as U/mg protein.

Catalase

Catalase activity was assessed according to Kinnula et al (Kinnula et al., 1992). The enzyme activity was measured polarographically by following oxygen production with a Clark oxygen electrode fitted into a stirred chamber. The cells were sonicated in Tris-EDTA (pH 7.4) and the reaction was started by adding the cells into the buffer containing 0.5 mM H_2O_2 equilibrated with nitrogen. The effect of catalase inhibition was tested with ATZ. The activity is expressed as units per mg of total cellular protein.

Glutathione content

Total glutathione content was determined spectrophotometrically following the reduction of 5,5'-dithiobis (2-nitrobenzoic) acid by NADPH in the presence of glutathione reductase (Beutler et al., 1975, Akerboom et al., 1981). Glutathione content is expressed as nmol/ mg protein. Proteins were analyzed by the micromethod of BioRad (Hercules, Ca, USA).

Immunohistochemistry

The biopsy material was first fixed in 10% neutral formalin, dehydrated and embedded in paraffin. Four micron thick sections were cut from a representative paraffin block, the sections were deparaffinized in xylene, and rehydrated in through a series of ethanol solutions. Endogenous peroxidase was consumed by incubating the sections in hydrogen peroxide in absolute methanol (0.3% for 30min, I; 0.1% for 10 minutes IV, V, VI). To enhance the immunoreactivity, the sections were incubated in 10 mM citrate buffer (pH 6.0) and boiled in a microwave oven for 2 min at 850 W and 8 min in 350 W. The paraffin blocks were then incubated with 2% milk powder to diminish background staining.

For immunocytochemistry (I), cells (MeT5A, M14K and M38K) were grown on microscopic slides (slidechambers, Nalge Nunc) using the same culture conditions as already described. The cells were air-dried and heat-fixed at 38°C for 1 h before further processing.
A modification of immunocytochemistry was applied to evaluate proliferation and apoptosis. In V the cell pellets from cell cultures were fixed in 10% neutral formalin overnight, after which formalin was removed, and melted 2% agar was laid over the pellets. The agar blocks were further embedded in paraffin. Four micron thick sections were cut from the cell blocks and processed further as described above.

MnSOD (I)

The polyclonal rabbit antibody for human MnSOD was a generous gift from Professor James D. Crapo (National Jewish Medical Center, Denver, Colorado). After blocking the endogenous peroxidase activity, the sections were incubated with the primary antibody overnight at room temperature. After incubation with a biotinylated swine anti-rabbit secondary antibody for 30 min, the avidin-biotin complex treatment for 30 min was performed. Diaminobenzine was used as a chromogen. The sections were lightly counterstained with haematoxylin and mounted with Eukitt. PBS or normal rabbit serum were used instead of primary antibody in negative controls.

γ-glutamylcysteine synthetase (IV,V)

The polyclonal rabbit antibodies for human γGCSh and γGCSl were kindly provided by Dr. Terrance Kavanagh (University of Washington, Seattle, Washington, USA). The sections were incubated with the primary antibodies for γGCSh and γGCSl, the immunostaining was done using the Histostain-Plus Kit (Zymed Laboratories Inc, South San Francisco, CA) and the chromogen was aminoethyl carbazole (AEC) (Zymed Laboratories Inc.) In negative controls the primary antibodies were substituted with PBS or non-immune rabbit serum.

P-gp, MRP1 and MRP2 (VI)

A mouse monoclonal IgG1 antibody to human P-gp (JSB1, Alexis Biochemicals), MRP1 (MRPm6, Alexis Biochemicals) and MRP2 (Alexis Biochemicals) were used in incubation of the tumor sections. The immunostaining was done as with γGCS immunohistochemistry, except that the primary antibody was replaced by non-immune mouse or rat serum when assessing negative control stainings.

Ki-67 as a marker of proliferative activity (V, VI)

Tumor cell proliferation was studied with a monoclonal mouse anti-human Ki-67 antibody (Zymed, San Francisco, CA, USA). Ki-67 was developed using diaminobenzidine as a chromogen, and the sections were lightly counterstained with haemotoxylin and mounted with Eukitt as with MnSOD.

Light microscopic evaluation of immunoreactivity (I, IV, V, VI)

A semi-quantitative assessment of the immunohistochemistry of γGCS, P-gp, MRP1 and MRP2 was employed by grading the staining as (0) negative, (1) < 33%, (2) 33-66%, (3) 67-100% of tumor cells showing positive staining. In MnSOD immunohistochemistry the staining was classified into five groups as (0) negative, (1) <25%, (2) 25-50%, (3) 51-75% and (4) >75%. The intensity of the immunostaining was graded as (0) negative, (1) weak, (2) moderate, and (3) strong (V), with the multidrug resistance proteins also (4), very strong, was used (VI).
The qualitative and quantitative immunostaining scores were added in order to get a combined score for the immunoreactivity. The score was divided into four main groups (V, VI);
- = no immunostaining; score 0
+ = weak immunostaining; scores 1-2
++ =moderate immunostaining, scores 3-4
+++ =strong immunostaining, scores 5-8
Two authors did the evaluation of immunostaining separately. In immunocytochemistry, the staining of MnSOD was assessed by grading the average staining intensity of the tumor cells in comparison with the negative controls and non-malignant mesothelial cells (I).
The results for Ki-67 immunostaining were also divided into groups as follows.
Weak staining= less than 5 % of cell nuclei positive
Moderate staining= 5-10 % of cell nuclei positive
Strong staining= 10-50 % of cell nuclei positive
Very strong staining= over 50 % of cell nuclei positive

Assessment of cytotoxicity

Lactate dehydrogenase release (I, II)

Lactate dehydrogenase (LDH) release was measured from MeT5A, M14K and M38K cells in order to evaluate lytic cell damage. The cells were first exposed to menadione or epirubicin (I) or H_2O_2 and epirubicin (II). LDH was measured spectrophotometrically using pyruvic acid as the substrate (Bergmayer & Bernt, 1974). Total cellular LDH was measured from cell lysates obtained by 1% Triton X-100 treatment. The results are expressed as percent of LDH released into the medium from the total LDH amount from the cells and the medium.

Nucleotide depletion (II, V)

Depletion of high-energy nucleotides was used because of the sensitivity of this method in the assessment of cell injury. The cells were incubated for 16 h with 0.1 mM [^{14}C] adenine (specific activity 51-55 mCi mmol $^{-1}$) to prelabel the high-energy nucleotides in intact cells. After labeling, the cells were washed three times and then exposed to menadione, epirubicin (I) or cisplatin (V). After the exposure the medium was collected and the cells were extracted with 0.4 M perchloric acid. Thin-layer chromatography was used to separate the purine nucleotides (ATP, ADP and AMP) both in the cell extract and the medium and the nucleotide catabolic products (xanthine, hypoxanthine, uric acid) in the medium. The results are expressed as per cent distribution of radioactivity (counts per min, cpm) between nucleotides in the cells, nucleotides leaked to the medium and catabolic products in the medium.

XTT-assay (III, IV)

In selected experiments a cell proliferation kit, the microculture tetrazolium dye calorimetric assay (XTT assay) was used to assess cell damage. This method was conducted using a commercial kit according to the instructions of the manufacturer (Boehringer, Mannheim, Germany). The cells were grown in a 96-well microtiter plate and exposed to cisplatin for 48 hours. After the exposure, 50 μl of labeling mixture was added to each well and the absorbance was measured with the microculture plate reader (Victor; 1420 Wallac Inc, Turku, Finland). Viability of the cells is expressed as relative absorbance.

Viability, apoptosis and cell proliferation

Viable cells were counted under the microscope after pretreatment with TNFα, exposure to epirubicin (III) and exposure to various concentrations of cisplatin in cell cultures pretreated with BSO (V). Apoptosis was assessed by the ApopTag (Oncor, Gaithesburg, MD, USA) *in situ* detection kit both in tissue biopsies and cultured cells. ApopTag detects the 3'-ends of the DNA fragments generated by apoptosis-associated endonucleases. The sections were dewaxed in xylene, rehydrated in ethanol and incubated with 20 μg/ml proteinase K at room temperature for 15 min. Endogenous peroxidase activity was blocked by 2% H_2O_2. After this, the slides were exposed to terminal transferase enzyme and digoxigenin-labeled nucleotides. The slides were then incubated with anti-digoxigenin antibody labeled with peroxidase. The color was developed with diaminobenzine-H_2O_2 and the samples were lightly counterstained with haematoxylin. Cells were defined as apoptotic, if the whole nuclear area of the cell labeled positively. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the tumor cells that could be found either singly or in groups. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 high power fields (HPFs) and this figure was divided by the number of tumor cells in the same HPFs. In addition apoptosis was assessed by Western blot analysis where caspase 3 cleavage was detected as already described. Caspase 3 is a cysteine protease acting as an executor enzyme in the late phase of apoptosis (Nunez et al., 1998).

Ki-67 was used in the assessment of tumor cell proliferation. The antibody reacts with Ki-67 nuclear antigen associated with cell proliferation (Table 4). The antigen is present throughout the cell cycle except in the resting cells in phase G0. The results for Ki-67 immunostaining were evaluated by counting the percentage of positive nuclei out of the total tumor cell population.

Statistical analysis

SPSS (7.5) for Windows was used for the statistical analysis. Comparisons between two independent groups were performed using independent samples t-test or Mann-Whitney U-test. With dependent groups paired samples t-test was used. Analysis of variance and Kruskal-Wallis test or Scheffe's *post hoc* test were used when comparing several independent groups. The significance of association was determined by Fisher's exact probability test, correlation analysis and two-tailed *t*-test. The survival analysis was performed by the Kaplan-Meyer curve and the survival in relation to the histochemical findings was assessed by the log rank, Breslow tests and Tarone-Ware tests. In the immunoreactivity evaluation, correlation coefficient obtained from the results of two investigators was established according to Cohen's kappa statistics (IV, V, VI) (Silcocks, 1983). P-value less than 0.05 was considered statistically significant.

RESULTS

Expression of antioxidant enzymes in mesothelioma cells (I)

In human healthy visceral pleura no MnSOD immunoreactivity could be detected in five of the six biopsies (Fig 1a, I). In one case the mesothelial surface of the visceral pleura showed a granular layer of immunoreactivity.

All seven biopsies from mesotheliomas showed moderate or intense immunoreactivity in the malignant cells (Fig 1b, 1c, I). The immunoreactivity ranged from moderate to intense within the same sample.

MnSOD was also detected in the mesothelial cell line MeT5A and the mesothelioma cell lines M14K and M38K. MeT5A cells, however, showed positive immunoreactivity only in cell clusters, whereas M38K cells showed intense staining in all cells. The protein expression of MnSOD was verified with Western blot -assay.

In addition to MnSOD, CuZnSOD, catalase and glutathione peroxidase were assessed in the cell lines by Northern blot analysis and specific activity measurement. These results indicated that M38K cells had a higher level of these enzymes when compared to MeT5A and M14K cells that they were also more resistant to menadione and epirubicin [Fig 7, Fig 8, (I) Table 1,(II)]

Protection of mesothelioma cells against hydrogen peroxide and epirubicin (II)

Total GST activity and the levels of GSH were higher in M38K cells than in MeT5A and M14K cells (Table 1, II). M38K cells were also most resistant to hydrogen peroxide, as judged both by LDH-release and by depletion of high-energy nucleotides (Fig 1, II). Aminotriazole (ATZ) to inactivate catalase and buthionine sulfoximine (BSO) to inhibit γGCS, the rate limiting enzyme in GSH synthesis, were used to study the importance of the hydrogen peroxide scavenging mechanisms catalase and glutathione redox cycle in oxidant and drug resistance. After ATZ pretreatment, the activity of CAT decreased by 85% in MeT5A, 82% in M14K and 89% in M38K cells. Pretreatment with 0.2 mM BSO decreased glutathione to undetectable levels in MeT5A and M14K cells. In M38K after 0.2 mM BSO pretreatment 15% of baseline glutathione remained, and the concentration of 1 mM BSO was chosen for further experiments (7% of baseline GSH left). ATZ or BSO alone in these concentrations did not cause nucleotide depletion in any of the cell lines and were considered non-toxic.

Pretreated MeT5A and M14K cells were exposed to 0.1 mM and M38K cells to 0.5 mM H_2O_2 for 4 h. In M14K cells only BSO pretreatment potentiated nucleotide depletion, whereas in MeT5A and M38K cells both ATZ and BSO pretreatment enhanced adenine nucleotide catabolism in H_2O_2 exposed cells (Fig 2, II).

Similar experiments were conduced with epirubicin. MeT5A and M14K cells were exposed to 0.1 μg/ml, and M38K to 0.5 μg/ml epirubicin for 48 h. BSO pretreatment potentiated epirubicin toxicity in all three cell lines when assessed by nucleotide depletion, whereas ATZ pretreatment had no effect on the cellular nucleotide levels in any of these cell lines (Fig 3, II).
We also found a higher level of GST-π protein in M38K cells compared to MeT5A and M14K cells, suggesting that this isoenzyme may be essential in the drug resistance of these cells (Fig 4, II)

Antioxidant mechanisms in mesothelioma cells in comparison to lung adenocarcinoma cells (III)

The inducibility of MnSOD was investigated in the mesothelioma cell line M14K and in the human lung adenocarcinoma cell line A549. A549 cells contained lower basal level of MnSOD than M14K cells, the activity being 1.8 $\pm$ 0.6 and 28.3 $\pm$ 5.8 U/mg protein, respectively. In A549 cells all the cytotoxic drugs tested with the exception of methotrexate caused a modest increase in the mRNA level of the 4-kb transcript of MnSOD, but TNF-α a much higher increase in both 1 kb and 4 kb transcripts (Fig 3, III). TNFα increased the level of MnSOD protein in both cell types (Fig 1, III), but none of the cytotoxic drugs tested (cisplatin, epirubicin, methotrexate or vindesin) had any effect. The specific activity of MnSOD was induced significantly by TNFα only in A549 cells (+524%) (Fig 2, III) but the cytotoxic drugs had no inducing effect on the specific activity in either cell type.
A549 cells were more resistant against menadione and epirubicin than M14K cells (Fig 4, III). The hypothesis that MnSOD induction may increase oxidant and drug resistance was tested in A549 cells. TNFα pretreated cells were exposed to either menadione (16 h) or epirubicin (48 h) at concentrations that caused the loss of approximately 50% of the control cells. MnSOD induction did not protect these cells against either of these exposures (Fig 5, III).
A549 cells were also more resistant against exogenous H_2O_2 than M14K cells, which suggested the importance of the protective role of catalase and/or glutathione. The specific activity of CAT was approximately twice as high in A549 cells than M14K cells, and total glutathione levels were approximately 2.5 times higher (Fig 7, III)**.** The depletion of CAT (with ATZ) or glutathione (with BSO) made A549 cells more vulnerable to oxidant injury caused by H_2O_2 but only BSO pretreatment had an effect on the toxicity caused by epirubicin. The hypothesis that glutathione-related mechanisms are important for the resistance of A549 cells was supported by Western blot studies showing that γGCSh was prominently expressed in A549 but not in M14K cells (Fig 7, III). Furthermore, depletion of GSH with BSO enhanced the injury in A549 cells when exposed to cisplatin (Fig 1, IV). The activity of total GST was somewhat lower in A549 than in M14K cells, suggesting that GST may not be involved in the oxidant resistance of these adenocarcinoma cells.

Expression of γGCS subunits in lung tumor and mesothelioma biopsies (IV, V)

Antibodies for the heavy and light subunits of γGCS were used in staining lung tumor biopsies and cell lines. These antibodies were shown to be applicable in the histochemical assessment of paraffin embedded tissues, and γGCSh and γGCSl subunits were expressed in lung malignancies (Fig 1, IV).

Both subunits of γGCS were investigated in five samples of non-malignant mesothelium and 34 mesothelioma tumor biopsies. Non-malignant mesothelium showed no expression of γGCSh or γGCSl in any of the samples (Fig 1, V). Most mesotheliomas (29/34) showed strong intensity of immunoreactivity for γGCSh. The combined qualitative and quantitative scores of immunostaining are shown in Table 1(V).
The intensity of γGCSl immunoreactivity was considerably weaker. Of the 34 cases, 12 cases showed no immunoreactivity with the light subunit antibody, strong/moderate staining was seen in 15 cases. The histologic type of mesothelioma had no statistically significant effect on γGCS reactivity even though the epithelial subtypes had a tendency to show stronger immunoreactivity. There was no correlation between either γGCSh or γGCSl immunoreactivity and patient survival.

Expression and possible role of γGCS in mesothelioma cells *in vitro* (V)

Seven mesothelioma cell lines were investigated for the expression of both γGCS subunits with Western blot analysis. Both subunits were detectable in all cell lines (Fig 2, V). The cell line most resistant to oxidants, M38K, contained the highest level of γGCSh.
In the nucleotide depletion assay, M38K cells were more resistant to cisplatin than M14K cells. Pretreatment with BSO to deplete intracellular glutathione potentiated the cisplatin-induced toxicity significantly (Fig 4, V).
Caspase 3 cleavage was used as an indicator of the apoptotic process. M14K cells exposed to 50 μM cisplatin showed caspase 3 cleavage within 48 h, which was not potentiated by BSO pretreatment. In M38K cells no apoptosis could be seen when assessed by caspase 3 cleavage after cisplatin exposure without or with BSO (Fig 5, V). Apoptosis was also assessed morphologically after the same exposures. Again cisplatin caused apoptosis in M14K cells but not in M38K cells, and apoptosis was not enhanced by BSO. Apoptotic index in M38K cells was very low (between 2.4% in controls and 4.5% in cisplatin exposed BSO treated cells).

The expression of P-glycoprotein and multidrug-resistance proteins 1 and 2 (MRP1 and MRP2) in mesothelioma tissue biopsies (VI)

Positive staining with the P-gp antibody was seen in 61 % of the mesothelioma tumor biopsies (n=36): 13 cases showed strong, five moderate, and four weak staining (Table 1, VI). The reactivity was mainly localized to the plasmamembrane. Epithelial and biphasic subtypes showed significantly more often strong immunoreactivity than did the sarcomatoid subtype (p=0.031). No positive staining was observed in non-malignant mesothelial cells.

Positive immunoreactivity for MRP1 was seen in 58 % of the cases. The staining was both membrane-bound and cytoplasmic. Equal incidences of strong, moderate and weak immunoreactivity for MRP1 were seen. MRP1 reactivity was weaker in sarcomatoid than epithelial or biphasic mesotheliomas (p=0.034). No immunostaining was seen in samples of non-neoplastic mesothelium (Fig 1, VI).

MRP2 antibody detected non-specific nuclear immunostaining in addition to membrane-bound immunoreactivity, but only membrane-bound staining was considered significant. 33 % cases showed positive reactivity and the staining was usually weak. Significant association was detected between MRP2 and moderate or strong P-gp staining ($p=0.021$), but no association was found between MRP2 and MRP1 reactivity ($p=0.41$). Sarcomatoid mesotheliomas showed significantly more often negative MRP2 staining than epithelial and biphasic ones ($p=0.024$). Non-neoplastic mesothelium was not stained with MRP2 (Fig 1, VI). No significant association was found with patient survival and expression of any of the multidrug resistance proteins studied ($p=0.135$, $p=0.09$ and $p=0.88$ for P-gp, MRP2 and MRP1, respectively). There was no association between these glycoproteins and tumor proliferation or apoptosis.

We also looked for an association between expression of γGCS and that of MRP1 or MRP2. There was a significant correlation between γGCSh and MRP2 expression ($p=0.048$), whereas the correlation between γGCSl and MRP2 was not significant ($p=0.35$). No correlation was seen between MRP1 and γGCSh ($p=0.67$) or γGCSl ($p=0.50$), either.

Table 5. The expression of antioxidant enzymes and related mechanisms in mesothelial cell line (MeT5A), mesothelioma cell lines (M14K, M38K), lung adenocarcinoma cell line (A549), non-malignant mesothelium and mesothelioma biopsies published by us or other laboratories. The results have been assessed at the level of mRNA (Northern blot, NB), protein (Western blot, WB), immunohistochemistry (IH), and/or the specific activity (SA) or concentration (c), when available. Nd = not done.

Antioxidant/ related mechanism	Mesothelial cell line (MeT5A)	Mesothelioma cell lines (M14K/ M38K)	Lung adenocarcinoma cell line (A549)	Non-malignant mesothelium	Mesothelioma tissue samples
MnSOD	+ (NB,WB,IH,SA)	+++ / +++ (NB,WB,IH,SA)	(+) (NB,WB,SA)	neg (IH)	+++ (IH)
CuZnSOD	+ (NB,SA)	++ / ++ (NB,SA)	nd	nd	nd
Catalase	+ (NB,SA)	++/ +++ (NB,SA)	+++ (SA)	(neg, Kahlos, 2001, IH)	(+++, Kahlos, 2001, IH)
GSH	+ (C)	+/ +++ (C)	+++ (C)	nd	nd
GPx	+ (NB,SA)	+(+)/ +(+) (NB,SA)	nd	nd	nd
GST	+ (WB,SA)	++/ +++ (WB,SA)	+ (SA)	(++, Dejmek, 98, Segers, 96, IH)	(++ (+), Dejmek, 98, Segers, 96, IH)
γGCSh	+ (WB)	++/ +++ (WB)	+++ (WB)	neg (IH)	+++ (IH)
γGCSl	+ (WB)	+/ + (WB)	+ (WB)	neg (IH)	++ (IH)
MRP1	nd	nd	nd	neg (IH)	++ (IH)
MRP2	nd	nd	nd	neg (IH)	+ (IH)
P-gp	nd	nd	nd	neg (IH)	++ (IH)
TRX	nd	(++/++, Kahlos, 2001 NB,WB)	(++, Soini, 2001, WB)	(neg, Kahlos, 2001, IH)	(+++, Kahlos, 2001, IH)
PRXI-VI	(+, Kinnula, 2001,WB)	(+ - ++, Kinnula, 2001, WB)	nd	(neg, Kinnula, 2001, IH)	(+-+++, Kinnula, 2001, IH)

DISCUSSION

MnSOD

The role of MnSOD in cancer biology is controversial. In the literature, it has been stated repeatedly that MnSOD activity is diminished in cancer cells and regaining the activity will eventually decrease the state of malignancy (Oberley & Oberley). However, increased MnSOD activities have been documented in some cancer types, at least glioma (Cobbs et al., 1996), renal carcinoma (Oberley et al., 1994) colorectal cancers (Janssen et al., 1998) and thyroid malignancies (Nishida et al., 1993). In some of these cancers, increased MnSOD level has been linked with poor survival (Janssen et al., 1998; Ria et al., 2001).

Many problems arise, however, when investigating the expression and role of MnSOD in cancer cells. The studies that have found increased MnSOD levels have been criticized by arguing that they have not included adequate controls or that they have not measured the specific activity of the enzyme (Oberley&Oberley). Then again studies evaluating the role of MnSOD in malignancy have often been done using gene transfection. The problem with transfection is that it does not mimic the actual *in vivo* situation, in which many antioxidants may be simultaneously induced. Transfection of the MnSOD cDNA alone results in accumulation of H_2O_2 and subsequent toxicity, which could lead to conclusions based on false assumptions. The balance of antioxidants is crucial in protecting the cell against various exogenous and endogenous oxidants (de Haan et al., 1996; Li et al., 2000; Thannickal & Fanburg, 2000). Conclusions have also been drawn from activity measurements directly in plasma samples from patients. Also tumor homogenates have been used to measure enzyme activities. Neither of these methods describes enzyme activity in a cancer cell. Tumor homogenates contain various cell types that are not always cancerous, like stroma. The problem with continuous cell lines is that the levels of AOEs usually decline in culture and selection of tumor cells also takes place during culture. The activity of an enzyme such as MnSOD in cultured cells may also be dependent on factors such as plating density, stage of cell growth, and constituents of the culture medium. It has to be noted that MnSOD is essential to living cells; knockout mice lacking the MnSOD gene are not viable (Li et al., 1995). Furthermore, transgenic animals expressing elevated lung MnSOD activity are more resistant to high oxygen tension than the control animals (Tsan, 2001).

In this study, the level of MnSOD was found to be significantly higher in mesothelioma cells than in healthy mesothelium or non-malignant MeT5A mesothelial cells *in vivo* and *in vitro*. In addition to immunohistochemistry, in which tumor samples where compared with non-malignant mesothelium, MnSOD was further investigated using northern blot and western blot analysis, and the specific activity of the enzyme was assessed. All these results confirm the hypothesis that MnSOD is overexpressed in human pleural mesothelioma.

In the present study MeT5A was used as a model of a non-malignant cell line. It, however, does not represent a primary mesothelial cell line, as MeT5A has been transformed with SV40 and it has qualities of a reactive mesothelium. MeT5A cells are nevertheless non-cancerous with typical mesothelial characteristics. Due to the difficulties in obtaining healthy mesothelium for culture of human mesothelial cell, MeT5A cells have been widely used for this purpose. Since reactive mesothelium *in vivo* and also the cell clusters of MeT5A cells showed positive immunoreactivity (Kahlos et al., 2000a), MnSOD cannot be used in the differential diagnosis between reactive mesothelium and mesothelioma. The differential diagnosis between mesothelioma and adenocarcinoma in pleura is difficult. Further studies have shown that MnSOD is higher in primary malignant mesothelioma than in other tumors metastasized to pleura (Kahlos et al., 2000a). Thus MnSOD may play role in the differential diagnosis of mesothelioma.

Studies on mesothelioma cell lines revealed that the cell line with the highest MnSOD activity (M38K) contained also the highest level of other antioxidants, like catalase and glutathione. M38K also turned out to be the most resistant of the mesothelioma cell lines. This supports the hypothesis that a simultaneous increase of many AOEs offers best protection. However, many mechanisms other than AOEs may also be overexpressed in this cell line, for example the apoptotic protein Bcl-X (Soini et al., 1999), DNA repair mechanisms, or even the drug efflux pumps.

It has been suggested that MnSOD cannot be induced in cancer cells (Wong & Goeddel, 1988). The present study, nevertheless, found that MnSOD protein and activity were increased by TNFα in both a mesothelioma cell line (M14K) and a pulmonary adenocarcinoma cell line (A549). This result is also in line with some other studies showing MnSOD induction in malignant cells at least by TNFα (Warner et al., 1996). In the present study MnSOD was induced more in A549 cells, in which the baseline MnSOD was low. Some of the cytotoxic drugs tended to increase MnSOD mRNA levels, but induction failed to reach significance. Induction *in vivo* is, however, possible. Other investigators (Akashi et al., 1996; Das et al., 1998) have shown that MnSOD mRNA was upregulated after anticancer drug treatment in cultured cells and that NF-κB is indeed induced by anticancer agents without TNFα involvement (Das & White, 1997). The induction *in vivo* is dependent on the drug concentration at the site of the tumor and time of exposure and these are difficult to mimic in cell cultures.

Induced MnSOD level has been suggested to offer protection against oxidant stress such as radiotherapy (Wong & Goeddel, 1988). Pulmonary adenocarcinoma cells treated with TNFα had an elevated level of MnSOD and increased resistance to paraquat (Warner et al., 1996). Its role in anticancer drug resistance has however, remained unclear and controversial. In this study, MnSOD induction failed to offer extra protection against either oxidants or the anti-cancer drug epirubicin. If anything, the cells were more susceptible to oxidant injury. The effects of TNFα are, however, numerous and TNFα has been shown to induce the apoptotic pathways by activating NF-kB (Darnay & Aggarwal, 1997). There is evidence that higher MnSOD level offers protection against apoptosis by blocking the effects of TNFα and the same effect has been ascribed to γGCS that inhibits the activation of NF-kB and prevents the apoptotic cascade (Manna et al., 1998; Morales et al., 1997; Obrador et al., 1997).

Several studies on cell cultures and experimental models and MnSOD transfected cells have suggested that MnSOD reduces tumor cell proliferation (Oberley&Oberley). Kahlos et al reported that high MnSOD in mesothelioma correlates with decreased proliferation. However, there was no relationship between proliferation or spontaneous apoptosis and patient survival (Kahlos et al., 2000b).
Based on previous and the present results, MnSOD is elevated in mesothelioma and may possibly be used as an additional marker in the differential diagnosis of this disease. MnSOD induction does not protect malignant cells against oxidant and drug exposure, and besides MnSOD, H_2O_2 scavenging AOEs may play an important role both in primary and acquired drug resistance.

Catalase

There are only a few studies on catalase in malignant tumors, and no major role has been proposed for it in anti-cancer drug resistance (Sinha & Mimnaugh, 1990; Tome et al., 2001). The present study showed that the mRNA and the specific activity of catalase were higher in the most resistant mesothelioma cell line compared to the non-malignant mesothelial cell line. In addition, a recent study confirmed the overexpression of catalase in mesothelioma. In that study a coordinate expression of catalase and MnSOD predicted a better survival of mesothelioma patients (Kahlos et al., 2001b). However, in cultured mesothelioma cells, inactivation of catalase with ATZ did not have significant effect on the toxicity of epirubicin, although it increased the susceptibility to H_2O_2-induced toxicity. This would imply a function for catalase during heavy oxidant exposure, which is in line with its physiological role (Halliwell&Gutteridge). On the other hand, ATZ does not completely inactivate catalase, so these studies may underestimate its role. Besides MnSOD and CAT, high oxidant resistance of mesothelioma may thus involve a simultaneous induction of many other antioxidant mechanisms most importantly the glutathione system.

Glutathione and GSH-related enzymes

The level of glutathione has often been linked with anti-cancer drug resistance (O'Brien & Tew, 1996; Tew, 1994; Zhang et al., 1998). Glutathione can confer resistance to cytotoxic drugs that cause ROS generation through the classical antioxidant pathway, the glutathione redox cycle. It is also irreversibly consumed in detoxification reactions catalyzed by GSTs. Also some of the plasmamembrane drug efflux pumps, mainly MRP1 and MRP2, are dependent on glutathione (Borst et al., 2000). Resistance to apoptosis has been suggested to be redox-regulated as well (Manna et al., 1998; Susin et al., 1998). Therefore, the synthesis and maintenance of the intracellular GSH level and the GSH:GSSG ratio are relevant for optimal defense against cytotoxic drugs.

M38K, the most resistant mesothelioma cell line, contained not only the highest level of MnSOD and catalase, but also of glutathione, glutathione peroxidase, glutathione S-transferase and γGCSh. Depletion of intracellular glutathione resulted in reversal of the resistance to epirubicin and cisplatin. Additional studies with the adenocarcinoma cell line A549 also emphasized the importance of the glutathione system both in the oxidant and drug resistance. A549 had a higher level of glutathione and γGCS compared to M14K. Inhibition of γGCS with BSO also enhanced the oxidant and drug (cisplatin) sensitivity of these cells significantly. A549 cell line is a resistant cell line to oxidants and oxidant producing drugs, but in these cells the MnSOD level was very low whereas GSH content was high. In light of these results, glutathione depletion with BSO could possibly have therapeutic implications in lung cancer and mesothelioma. One way of reversing the resistance could be injection of BSO intrapleurarly to avoid the systematic problems of glutathione depletion. In fact, phase I trials have been conducted with BSO and melphalan, and the continuous-infusion BSO was relatively nontoxic and resulted in depletion of tumor glutathione (Bailey et al., 1997).

The capacity to synthesize GSH is essential in protection and detoxification reactions of non-malignant and malignant cells. In addition to cysteine availability, the expression and activity of γGCS is of major importance in maintaining adequate levels of GSH. Most articles regarding γGCS have been studies on γGCSh and its regulation, lately some studies have been published also on the light subunit (Galloway et al., 1997; Shi et al., 1994; Tipnis et al., 1999; Zhang et al., 1998). Both subunits of γGCS have been connected with resistance against melphalan, doxorubicin and cisplatin (Iida et al., 1999). No studies have, however, been done with the actual expression and distribution of the enzyme subunits in malignant tumors. There is a recent microarray study comparing a mesothelioma cell line with a non-malignant mesothelioma cell line showing increased expression of γGCS in the malignant cell (Rihn et al., 2000). Yet only mRNA, not protein, was detected from these cells.

In the present study all mesothelioma cell lines expressed both subunits of γGCS. The highest level of γGCSh, which is responsible for the catalytic activity, was found, not surprisingly, in the M38K cells. In immunohistochemistry most mesotheliomas expressed both subunits of γGCS. Healthy mesothelium was negative, but by immunohistochemistry the presence of small amounts of the enzyme in healthy tissue cannot be excluded. No correlation between patient survival and γGCS expression was observed. On the other hand, the overall prognosis of mesothelioma is poor and the survival time is not always reliable as the time of diagnostic biopsy within the time span of the disease varies uncontrollably between patients. Results from a recent immunohistochemical study with lung cancer biopsies also showed that both subunits of γGCS are abundantly expressed in lung cancer cells (Soini et al., 2001b). In these samples a positive correlation was found between high apoptotic tendency and low γGCS expression. These results are in line with the previous report that γGCS is antiapoptotic as it changes the redox-state of the cell possibly by inhibiting the pro-apoptotic signals caused by e.g. TNFα (Manna et al., 1998). This same result was not achieved with the mesothelioma biopsies. In the present study BSO pretreatment of mesothelioma cell lines that was followed by cisplatin exposure did not enhance apoptosis in M38K cells, indicating strong resistance towards apoptosis but also other mechanisms than the redox state in controlling the apoptotic pathway.

GST-π has been reported to be overexpressed in neoplastic tissues (O'Brien & Tew, 1996). Also the relationship between the overexpression of GSTs and the sensitivity of tumor cells has been documented (Hao et al., 1994, reviewed by Zhang et al., 1998). The present study found a higher expression of GST-π in the M38K cell line than the more sensitive mesothelioma cell line and the non-malignant cell line. In the adenocarcinoma cell line the activity of total GST was considerably lower compared to the mesothelioma cell lines. On the other hand, A549 cells were more resistant to oxidants and cytotoxic drugs and had a significantly higher GSH-level than mesothelioma cells. These results are in line with others showing that GSH and its re-synthesis capacity explain the resistance of A549 cells to cytotoxic drugs (al-Kabban et al., 1990), cadmium (Hatcher et al., 1995) and oxidants (Arsalane et al., 1997; Rahman et al., 2001). On the other hand, there are studies showing that lung cancer cell lines can express several GSTs and GPx without a GSH increase (Hao et al., 1994). Multiple detoxification mechanisms may thus be involved and non-enzymatic detoxification by glutathione conjugation may take place as well. The resistance mechanisms must also differ between different drugs since their way of killing the cancer cells is diverse. Anthracyclins that cause ROS formation are possibly detoxified in part by AOEs. On the other hand, cisplatin may not cause oxidative stress and thus it may be conjugated to GSH and exported from the cell; the role of MnSOD and other antioxidants in cisplatin resistance may therefore be minimal.

The induction of the salvage enzyme γGT has been reported during the development of drug resistance. In a study analyzing 451 human cancers, all 10 mesothelioma samples were negative for γGT immunoreactivity (Hanigan et al., 1999). This suggests that intracellular glutathione levels are maintained with *de novo* synthesis probably by γGCS.

To conclude, the present study emphasized the importance of GSH-related mechanisms in the intrinsic resistance of mesothelioma, and these findings were supported by studies conducted with lung adenocarcinoma cells. High expression of γGCS is of major importance both in the detoxification pathway and the antioxidant function of these cells.

P-gp and MRP1&2

The plasmamembrane pumps have been extensively studied, and the emphasis has been on P-gp (Pastan & Gottesman, 1987). One study on cultured mesothelioma cell lines suggested that the resistance to vindesin and doxorubicin is P-gp dependent. In that study, however, the baseline expression of P-gp was almost undetectable, and the expression increased when exposing the cells continuously to the cytotoxic drugs (Licht et al., 1991; Licht et al., 1995) Therefore, P-gp is not likely to account for the primary resistance of the tumor. In an immunohistochemical study on 33 mesothelioma tumor biopsies, P-gp was expressed in the majority of tumors (Ramael et al., 1992). In another study on the expression of P-gp, MRP1 and γGCS in five mesothelioma cell lines the conclusion was that MRP1 and γGCS may be co-ordinately overexpressed in doxorubicin-resistant cells (Ogretmen et al., 1998).

The present study found the expression level of P-gp to line up with findings from other tumors, such as kidney, colon and liver (Duensing & Slate, 1994; Sinicrope et al., 1992; Soini et al., 1996). MRP1 was expressed in the majority of the biopsies, which is in agreement with other tumors such as colon and gastric cancers (Endo et al., 1996; Fukushima et al., 1999). MRP2 was expressed in only 33% of mesotheliomas, and the staining was usually weak. It has been detected in unselected lung, gastric and colorectal tumor cell lines (Kool et al., 1997; Taniguchi et al., 1996). None of these proteins correlated with patient survival so the expression of these efflux proteins cannot be used as a prognostic factor. A recent study has suggested that even a low expression of these transport proteins may cause clinical drug resistance, as the maximum dose of a cytotoxic drug tolerated by the patient is often barely sufficient to kill a useful percentage of the tumor cells (Allen et al., 2000). Therefore relatively small increases in the drug resistance in tumor cells are sufficient to make the drug clinically ineffective. Our results show that these transporters cannot be used for the assessment of primary resistance, as most patients did not receive chemotherapy and the efflux pumps are not known to cause proliferation of the malignant cells or progression of the disease. In agreement with this neither tumor proliferation nor apoptosis showed any association with the expression of the multidrug resistance proteins.

γGCS expression correlated with MRP2, but not with MRP1. A connection has been made with γGCS and MRP1 in a number of studies (Ishikawa et al., 1996; Kuo et al., 1996) but no corresponding studies on MRP2 are available. MRP2 has not, however, been studied as much and even its expression in different malignancies is largely unknown. Cisplatin has been shown to cause upregulation of MRP2, and MRP2 to cause resistance against cisplatin (Borst et al., 2000; Konig et al., 1999). Most of our patients had not received chemotherapy so the immunoreactivity of MRP2 describes primary, not cisplatin induced, expression of the protein. (Ishikawa et al., 1996. It remains to be investigated whether this correlation has any true significance in resistance of mesothelioma, as the expression of MRP2 is weak altogether and present only in 33% of the biopsies. In contrast of trying to find significant associations with patient survival, the expression of these mechanisms might be considered as tumor characteristics that can be used when trying to understand the natural course of this malignancy. In fact the actual role of these efflux proteins in the clinical primary resistance of any malignant tumor is far from clear. For example, new members to the MRP family have been discovered (Bera et al., 2001) and their contribution to clinical drug resistance has not yet been studied.

CONCLUSIONS

Malignant pleural mesothelioma shows a simultaneous high expression of several antioxidant enzymes and related proteins. These mechanisms may play an important role in the overall poor prognosis, and in the drug resistance of this disease. The following conclusions can be drawn from the present study:

1. The level of MnSOD is significantly higher in malignant mesothelioma cells than in non-malignant mesothelial cells both *in vitro* and *in vivo*. High MnSOD can be detected at the level of mRNA, immunoreactive protein and specific activity.

2. MnSOD in mesothelioma cells can be induced by TNFα but not by the cytotoxic drugs. Induction of MnSOD, however, does not provide any protection against repeated oxidant or drug exposures. Additional studies with lung adenocarcinoma cells further suggest the importance of high glutathione content in the oxidant and drug resistance of these cells.

3. The resistance of mesothelioma cells *in vitro* is partly associated with glutathione and glutathione S-transferase whereas the significance of catalase is limited only to heavy oxidant exposure.

4. In addition to MnSOD, γGCS, the rate-limiting enzyme in glutathione synthesis, is expressed at high quantities in most mesotheliomas. γGCS may play role in the overall primary drug resistance of malignant lung cells, since treatment of mesothelioma and lung adenocarcinoma cells with BSO, to inhibit γGCS, significantly potentiates drug-induced cytotoxicity.

5. Variable expression of P-gp, MRP1 and MRP2 can be detected in malignant mesothelioma, suggesting that the primary resistance of mesothelioma is not solely dependent on their expression or function. As tumor growth, apoptosis or patient survival do not associate with these membrane glycoproteins, they cannot be used as a prognostic factor in mesothelioma.

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